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## CLINICAL AND EXPERIMENTAL

### INFECTIVITY AND INTERRELATIONSHIPS OF 2060 AND JH VIRUSES IN VOLUNTEERS


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THE two viruses known as 2060 and JH were recovered in monkey kidney cell tissue cultures from persons having acute afebrile coryzal respiratory illnesses.[1, 2] This association with clinical illness suggested that they might be etiologically related to the common cold. Indeed, a study by Price[3] showed that children were protected from the common cold by immunization with JH vaccine. On the other hand, the causative role of these viruses in clinical respiratory illness cannot be considered certain, and the postulates for pathogenicity which require viruses to satisfy the classical criteria of Koch for the identification of pathogenic bacteria[4, 5] have not been entirely fulfilled by published data.

Studies in volunteers were undertaken to elicit the infectivity of tissue culture harvests of these viruses and the clinical illness and the serologic response to a specific challenge. The interrelationship between the two agents as well as their relationship to infectious nasal secretions collected from donors with a natural common cold was examined.

#### MATERIALS AND METHODS

*Volunteers.*—The subjects were young adult students in the colleges of nursing, medicine, and dentistry. Nearly all of them were 21 to 25 years of age and the extremes were


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18 and 36 years of age. The selection and observation of the volunteers, the method of challenge, and the criteria of an experimental cold have been described in detail in another report.[6] The occurrence and severity of sneezing, sore throat, nasal discharge, nasal obstruction, cough, chilliness, headache, and malaise were recorded for each 24 hour period before, and for 6 days after, the experimental challenge. The decision as to whether or not a volunteer developed a cold after the challenge was based upon the total symptom score, the duration and severity of nasal discharge, and the subject's statement as to whether he had a cold.

*Viruses.*—The fifth or sixth subpassages of the 2060 virus, and the thirteenth or fourteenth passage of JH virus* in monkey kidney cell tissue cultures were used. Prior to the challenge of volunteers, the tissue culture harvests were shown to be nonpathogenic for rabbits, mice, suckling mice, and embryonated eggs after several different routes of inoculation. Neither did they have a cytopathogenic effect upon HeLa cells in tissue culture. The titer of the 2060 virus was approximately $10^3$ and that of the JH virus approximately $10^{3.5}$ fifty per cent infectious doses for monkey kidney cells in tissue culture.

For the experimental challenge a harvest of infected tissue culture fluid was diluted 1:2, 1:10, or 1:100 with sterile Hanks' solution, and 0.2 ml. was instilled into each nostril of the volunteers. Control subjects received either a buffered salt solution or tissue culture fluid harvested from uninoculated monkey kidney cell cultures. Other volunteers were challenged with diluted nasal secretions obtained from donors who had a characteristic common cold.[6] These were shown to be free from known respiratory viruses by inoculation of tissue cultures, embryonated eggs, and appropriate animals.

*Virus Reisolation.*—Recovery of the virus from volunteers after challenge was made from swab specimens obtained from the nares. The specimens were suspended in Hanks' salt solution and inoculated onto monolayers of primary monkey kidney cells in tubes. The inoculated tissue cultures were incubated at 37° C. in a roller drum. From each original sample 0.25 ml. was inoculated into each of 4 to 8 tubes, and all of the specimens were passed serially at least 3 times. All studies were done under blind code and included specimens from control subjects. The viruses recovered were neutralized by anti-JH rabbit serum. One of the reisolated viruses was used to challenge new volunteers.

*Neutralization Tests.*—The titers of neutralizing antibody against the initial and reisolated viruses in the prechallenge- and convalescent sera of volunteers were made in primary and secondary monkey kidney cell cultures. Second generation cultures were used in most experiments because of the enhanced degree of cytopathogenic effects. The medium used was either Mixture 199 or ½ Eagle's and ½ Mixture 199. The pH was maintained between 7.0 and 7.2 throughout all phases of the determination. In some tests the cells were chilled at 4° C. for 1 hour and then rewarmed to room temperature before inoculation. Paired acute and convalescent sera were always tested simultaneously under a code which did not indicate which of the 2 viruses was given or whether the subject received only a buffered salt solution. One hundred to 1,000 $TCID_{50}$ of virus was added to equal volumes of diluted sera, previously inactivated at 56° C. for 30 minutes. After holding at 4° C. in one laboratory and at 37° C. in the other for 30 minutes, 2 to 4 monkey kidney cultures were inoculated with 0.1 ml. of each dilution of virus-serum mixture. Simultaneous virus infectivity and serum controls were used. The inoculated cells and control cultures were examined microscopically at 1 to 3 day intervals for 7 to 10 days. End points were calculated from the results obtained at the time of appearance of definite cytopathogenic effects in control cultures inoculated with virus. Titers of neutralizing antibodies were expressed as the reciprocal of the initial dilution of the serum that effected definite inhibition of cytopathogenicity which was usually maintained for 48 hours.

## RESULTS

The cumulative results of 9 separate experiments are given in Table I. Ninety volunteers were challenged with 2060 virus and 56 simultaneous control

*Obtained from Dr. Winston Price.

TABLE I. NUMBER OF COLDS DEVELOPING IN SUBJECTS EXPERIMENTALLY INFECTED WITH 2060 AND JH VIRUSES

| INITIAL CHALLENGE* | NO. VOLS. | EXPERIMENTAL RESULT | | | SIGNIFICANCE OF THE DIFFERENCE (VIRUS VERSUS BUFFER) |
|---|---|---|---|---|---|
| | | NO COLD | COLD | PER CENT COLDS | |
| 2060 | 90 | 65 | 25 | 28 | >0.05 |
| Buffer | 56 | 47 | 9 | 16 | |
| JH | 69 | 46 | 23 | 34 | >0.05 |
| Buffer | 40 | 32 | 8 | 20 | |
| 2060 or JH | 159 | 111 | 48 | 30 | <0.05 |
| Buffer | 96 | 79 | 17 | 18 | |

*Four - 14 × $10^3$ monkey kidney tissue culture $ID_{50}$.

subjects were given uninfected tissue culture fluid or buffered salt solution. Sixty-nine persons received JH virus and 40 others, serving as controls, received buffer solution in these experiments. The inoculum of JH virus contained 4,000 to 14,000 fifty per cent monkey kidney tissue culture infectious doses of virus. Twenty-five, or 28 per cent, of the 90 volunteers challenged with 2060 developed an experimental cold. Although this was a greater proportion than the 16 per cent of control subjects who developed a cold, the difference was not statistically significant.

Among the 69 volunteers challenged with JH virus, 23, or 34 per cent, developed an experimental cold. Among the 40 simultaneous control subjects 8, or 20 per cent, had a cold. This difference in colds is not statistically significant.

If the results with the 2 viruses are added, 30 per cent of 159 challenged volunteers developed an experimental cold, as compared to 18 per cent of 96 control subjects. In these cumulative results the tissue culture harvests with virus were significantly more infectious than the control inoculums.

The rank order of occurrence of 8 clinical symptoms after challenge with 2060 or JH virus is shown in Fig. 1. The relative frequency and severity of each of the symptoms is demonstrated on the ordinate by the mean specific symptom score. The illness that followed administration of either of the viruses was characterized mainly by coryzal upper respiratory symptoms. More than one half of the total symptom score was from nasal discharge and nasal obstruction; approximately one third was contributed by sneezing, sore throat, and cough. Constitutional symptoms were definitely of lesser severity. Although JH virus tended to produce a slightly higher frequency of symptoms, the clinical response to the 2 viruses was nearly identical.

TABLE II. ACQUIRED CROSS IMMUNITY BETWEEN 2060 AND JH VIRUSES

| INITIAL CHALLENGE | | | RECHALLENGE | | | SIGNIFICANCE OF THE DIFFERENCE (INITIAL VERSUS RECHALLENGE) |
|---|---|---|---|---|---|---|
| AGENT | NO. VOLS. | % COLD | AGENT | NO. VOLS. | % COLD | |
| 2060 | 11 | 55 | 2060 | 11 | 9 | <0.05 |
| 2060 | 14 | 22 | JH | 14 | 0 | Not significant |
| JH | 15 | 40 | JH | 15 | 7 | <0.05 |
| JH | 15 | 40 | 2060 | 15 | 7 | <0.05 |
| Either | 55 | 38 | Either | 55 | 5 | <0.001 |

The incubation period after challenge until the onset of symptoms usually was less than 24 hours for either of the viruses, as shown in Fig. 2. The peak of the frequency and severity of symptoms occurred 48 to 72 hours after challenge with JH virus, and 96 to 120 hours after 2060 virus. The symptoms subsided quickly after the zenith of illness was reached. The average total symptom score of volunteers who were challenged with virus and did not develop a cold was no higher than the score for the control subjects.

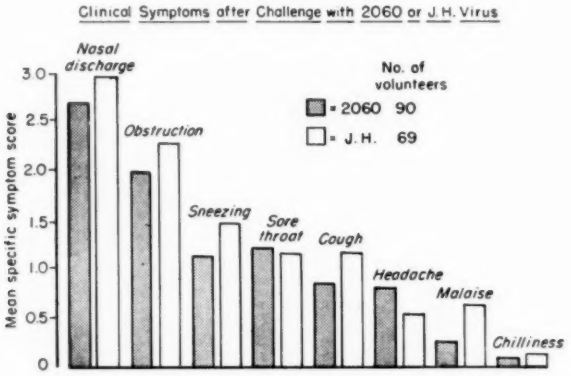


Fig. 1.

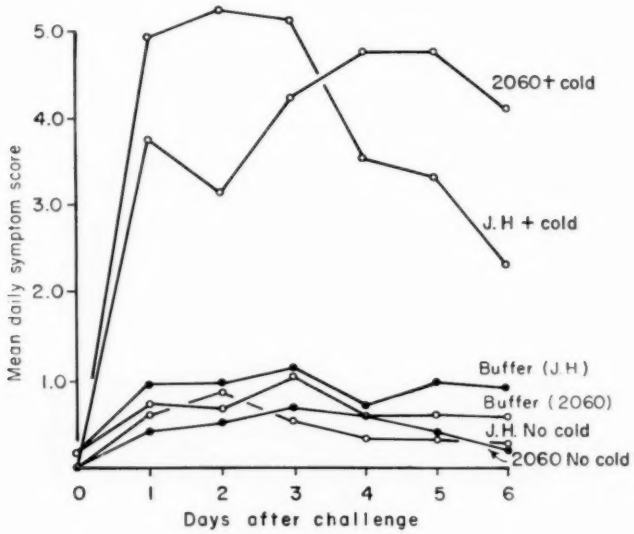


Fig. 2.

Fifty-five volunteers who received 2060 or JH virus were rechallenged with the same or the other virus 4 weeks to 8 months after the initial challenge. The results are shown in Table II. Among 11 volunteers initially challenged with 2060 and later rechallenged, 6, or 55 per cent, developed a cold in the first experiment and only 1, or 9 per cent, after the second challenge with 2060. Among 14 volunteers who initially received 2060, which caused 3, or 22 per cent, colds, a later challenge with JH failed to produce any colds. Among

30 volunteers to whom JH virus was given in the first of 2 experiments, 12, or 40 per cent, developed a cold from the first challenge. Rechallenge of 15 of them produced only 1 cold, or 7 per cent. Challenge of the other 15 volunteers with 2060 likewise produced only 1 cold. Thus, only 3, or 5 per cent, of 55 subjects developed colds from the second challenge with 1 of the 2 viruses, whereas 38 per cent of the same subjects had a cold after the initial challenge. These differences are highly significant. They show both acquired immunity and effective cross immunity between the 2 agents.

In contrast to the evidence for effective cross immunity between 2060 and JH viruses acquired from previous challenge with one of the agents, a previous challenge with infectious nasal secretions from donors with a natural common cold conferred no immunity to 2060 or JH (Table III). This would suggest that the infectious agents of natural common colds previously studied[6, 7] are other than 2060 or JH viruses.

In 1 experiment, recovery of virus from nasal secretions of 17 volunteers was attempted prior to challenge, and on Days 2, 4, and 6 after administration of JH virus. The results are given in Tables IV, V, and VI and Fig. 3. The virus was recovered from the challenge inoculum on the day of the experiment

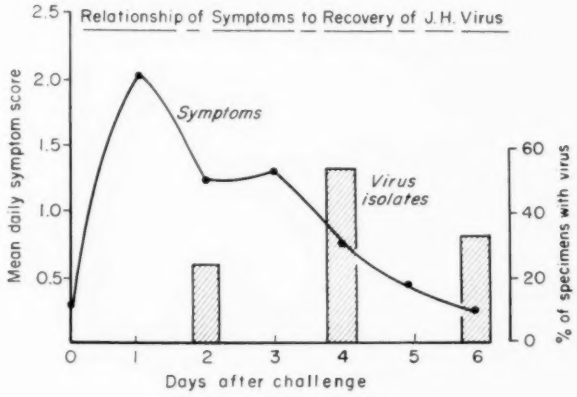


Fig. 3.

and after 2 days of in vitro incubation at 37° C., but not on Days 4 and 6. As shown in Table IV, no virus was recovered from prechallenge nasal secretions. JH virus was reisolated from 36 per cent of 47 specimens collected from volunteers within 6 days after the challenge. This included 24 per cent on Day 2, 53 per cent on Day 4, and 33 per cent on Day 6. Positive isolates were obtained from all of 5 specimens taken from the 2 subjects who developed an experimental cold and from 29 per cent of the 42 specimens from 15 volunteers who received the virus, but did not develop symptoms of a cold (Table IV).

The relationship of the isolation of virus and the presence of clinical symptoms among these subjects is shown in Fig. 3. Detectable virus in the secretions lagged behind the symptoms and was most prevalent 4 days after challenge at a time when the symptoms were declining.

TABLE III. LACK OF CROSS IMMUNITY BETWEEN 2060 OR JH VIRUS AND OTHER INFECTIOUS NASAL SECRETIONS

| RECHALLENGE | PREVIOUS CHALLENGE | NO. OBSERVED | % COLDS |
|---|---|---|---|
| 2060 | No previous challenge | 72 | 25 |
| | Infectious nasal secretion | 18 | 39 |
| | 2060 or JH virus | 26 | 8 |
| JH | No previous challenge | 49 | 35 |
| | Infectious nasal secretion | 21 | 33 |
| | 2060 or JH virus | 29 | 4 |

TABLE IV. RECOVERY OF JH VIRUS FROM VOLUNTEERS IN RELATION TO ILLNESS

| EXPERIMENTAL RESULT | VIRUS ISOLATES* | | | | SPECIMENS TAKEN | |
|---|---|---|---|---|---|---|
| | PRE-CHALLENGE | DAY 2 | DAY 4 | DAY 6 | NO. | % POSITIVE |
| Cold | 0/2 | 2/2 | 1/1 | 2/2 | 5 | 100 |
| No cold | 0/15 | 2/15 | 7/14 | 3/13 | 42 | 29 |
| % virus isolates | 0 | 24 | 53 | 33 | 47 | 36 |

*Numerator denotes the number of positive isolates; denominator denotes the number of subjects whose specimens were examined.

Proliferation of virus in both symptomatic and nonsymptomatic volunteers was evident. (Table V.) Virus was present in the secretions of 3 asymptomatic subjects on Day 4 only; 3 others had virus isolates on 2 of the 3 days on which specimens were taken. Virus was consistently present on Days 2, 4, and 6 in the secretions of 3 other volunteers; 2 of these developed a clinical cold and 1 did not. The virus isolated on Day 2 from the latter person without symptoms was harvested and shown to be neutralized by anti-JH rabbit serum. The second tissue culture passage of this isolate was then used to challenge 6 new volunteers, and produced experimental colds in 2 of them.

TABLE V. PERSISTENCE OF JH VIRUS IN NASAL SECRETION FROM VOLUNTEERS

| DAY AFTER CHALLENGE ON WHICH VIRUS WAS RECOVERED | VOLUNTEERS STUDIED (NO.) | VOLUNTEERS FROM WHOM VIRUS WAS REISOLATED (NO.) | | VOLUNTEERS WITH VIRUS (%) |
|---|---|---|---|---|
| | | COLD | NO COLD | |
| 2 | 17 | 0 | 0 | 0 |
| 4 | 15 | 0 | 3 | 20 |
| 6 | 15 | 0 | 0 | 0 |
| 2 and 4 | 15 | 0 | 1 | 7 |
| 4 and 6 | 13 | 0 | 2 | 15 |
| 2, 4, and 6 | 13 | 2 | 1 | 23 |
| Any day | 17 | 2 | 7 | 53 |

TABLE VI. RECOVERY OF JH VIRUS FROM VOLUNTEERS IN RELATION TO PRIOR CHALLENGE

| VOLUNTEER STATUS | | NUMBER OF VOLUNTEERS | | NUMBER OF SPECIMENS | |
|---|---|---|---|---|---|
| PRIOR CHALLENGE WITH 2060 OR JH VIRUS | RESULT OF PRIOR CHALLENGE | VIRUS ISOLATED/TOTAL OBSERVED | % POSITIVE | VIRUS ISOLATED/TOTAL OBSERVED | % POSITIVE |
| Yes | Cold | 0/4 | 0 | 0/10 | 0 |
| Yes | No Cold | 4/6 | 67 | 6/18 | 33 |
| No | — | 5/7 | 72 | 11/19 | 59 |

That the recovery of virus was influenced by prior exposure to JH or 2060 virus is shown in Table VI. Examination of 10 specimens from 4 persons who had previously developed an experimental cold from 1 of these agents yielded no virus isolates. Among volunteers who had been challenged previously with 2060 or JH but who did not develop a cold as a result of that challenge, $\frac{1}{3}$ of 18 specimens contained virus, and $\frac{2}{3}$ of the subjects had at least 1 positive isolate. Among persons who had no previous experimental exposure to 2060 or JH virus, 59 per cent of 19 specimens and 72 per cent of subjects yielded JH virus during the experiment. All of the reisolated viruses were neutralized by anti-JH rabbit serum.

As shown in Table VII, all subjects from whom virus was obtained and on whom paired specimens of serum were available had a fourfold or greater rise in serum neutralizing antibody titer for the specific reisolated virus. The convalescent serum specimens were collected approximately 3 weeks after the challenge. The initial titer in the subjects who developed a cold was 1:4 and 1:8; the initial titer in the others ranged from < 1:2 to 1:64. In the convalescent serum all but one subject had a titer greater than 1:8. Cross neutralization of heterologous and stock JH virus was of lesser degree than with homologous virus.

The results of neutralization tests performed with no knowledge of the derivation of 91 paired specimens of sera obtained before challenge and 3 weeks later are shown in Table VIII. Among subjects who were challenged with either of the viruses, 8 of 44 or 18 per cent showed a fourfold or greater rise in antibody titer to 2060 virus. None of 18 pairs of sera from subjects who were challenged with buffer solution showed a rise in anti-2060 titer. Tests for neutralizing antibody against JH virus were run on 68 pairs of sera from volunteers challenged with either of the 2 viruses; 17, or 25 per cent, showed a fourfold or greater rise in anti-JH titer. Again, none of 19 pairs of sera collected from subjects who received buffer solution showed a fourfold rise in titer against this virus. A fourfold rise in neutralizing antibody against both viruses occurred in 5 of 41 pairs of sera or 12 per cent of volunteers who received one or the other of the viruses.

TABLE VII. THE ANTIBODY RESPONSE OF VOLUNTEERS TO THEIR OWN VIRUSES WHICH WERE REISOLATED AFTER CHALLENGE

| SUBJECT | CLINICAL COLD | NO. OF TIMES VIRUS WAS REISOLATED / NO. OF SPECIMENS TESTED | NEUTRALIZING ANTIBODY TITER / ACUTE SERUM | NEUTRALIZING ANTIBODY TITER / CONVALESCENT SERUM |
|---|---|---|---|---|
| JJo | Yes | 3/3 | 1:4 | 1:16 |
| MJo | Yes | 2/2 | 1:8 | 1:128 |
| Br | No | 3/3 | 1:16 | 1:64 |
| Rog | No | 2/3 | Not Done | Not Done |
| Co | No | 2/3 | 1:4 | 1:16 |
| No | No | 2/3 | 1:64 | 1:256 |
| Rod | No | 1/3 | <1:2 | 1:8 |
| Gi | No | 1/3 | 1:16 | 1:64 |
| Ow | No | 1/3 | 1:32 | 1:128 |

TABLE VIII. SEROLOGIC RESPONSES OF VOLUNTEERS TO AN EXPERIMENTAL CHALLENGE WITH 2060 AND JH VIRUSES

| CHALLENGE | SUBJECTS WHO DEVELOPED A COLD | | | | SUBJECTS WHO DID NOT DEVELOP A COLD | | | | TOTAL SUBJECTS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NO. STUDIED | NO. OF PAIRS TESTED AGAINST: | | | NO. STUDIED | NO. OF PAIRS TESTED AGAINST: | | | NO. STUDIED | NO. OF PAIRS TESTED AGAINST: | | |
| | | 2060* | JH* | BOTH* | | 2060* | JH* | BOTH* | | 2060* | JH* | BOTH* |
| 2060 | 9 | 2/9 | 2/8 | 1/8 | 24 | 3/24 | 4/22 | 1/22 | 33 | 5/33 | 6/30 | 2/30 |
| JH | 7 | 0/2 | 2/7 | 0/2 | 31 | 3/9 | 9/31 | 3/9 | 38 | 3/11 | 11/38 | 3/11 |
| Either virus | 16 | 2/11 | 4/15 | 1/10 | 55 | 6/33 | 13/53 | 4/31 | 71 | 8/44 | 17/68 | 5/41 |
| Buffer | 5 | 0/5 | 0/4 | 0/4 | 15 | 0/13 | 0/15 | 0/12 | 20 | 0/18 | 0/19 | 0/16 |

*Numerator denotes the number of pairs of sera in which a fourfold or greater rise in antibody titer was observed between the prechallenge specimen and the convalescent specimen collected 3 weeks after challenge; denominator denotes the total pairs observed.

A rise in antibody occurred only slightly more frequently in subjects who developed a cold after challenge (5 of 16) than in those who did not (15 of 55). There was no significant difference in the initial antibody titer against 2060 or JH in volunteers who developed symptoms after challenge and those who did not. Volunteers in the buffer control group who developed a cold had no significant rise in antibody for these 2 viruses.

## DISCUSSION

Because the symptoms of a common cold can be produced by many different viruses, it is important to determine whether a virus isolated from a patient with symptoms of the common cold will produce colds consistently in human subjects. The clinical results obtained when we challenged volunteers with 2060 or JH virus did not answer this question conclusively, although they suggest that these viruses did cause the syndrome of the common cold. Although 30 per cent of the subjects developed a cold according to the criteria previously developed[6] in experiments on volunteers, 18 per cent of the subjects who concomitantly were given buffer solution as a control developed colds according to the same criteria. Since the subjects were not isolated, it is assumed that the respiratory infections that occurred in the control subjects resulted from contact with persons in the environment who were harboring one of the viruses that produce common colds. When the results of a large number of our previous experiments were added, we found that 10 per cent of subjects challenged with a buffer solution had symptoms of a common cold, but that this percentage varied in the different weeks of individual experiments. Because of the relatively high proportion of control subjects with colds observed in the experiments represented in this paper, a significantly greater number of colds among the subjects challenged with infectious solution can be demonstrated only by observation of a larger number of subjects. Nevertheless, the tissue culture harvests of 2060 and JH viruses have a relatively low clinical infectivity. Tyrrell and Bynoe[7] observed only 6 colds among 58 volunteers who were challenged with JH virus.

In the present series of experiments other criteria were used to determine whether the viruses proliferated and caused colds in the subjects who received them. In a previous publication[8] it was reported that 42 per cent of subjects who were challenged with an infectious nasal secretion for the first time developed a cold, as compared with 9 per cent of subjects who developed colds among those who were rechallenged with the same secretion after 3 to 45 weeks. On the basis of related data that showed neutralizing antibodies in the gamma globulin fraction of serum[9] this was interpreted to mean that humoral immunity had developed against a virus contained in the infectious secretion. The same phenomenon is reported in the present article. Among subjects who received 2060 or JH virus, 38 per cent developed colds after the initial challenge with either of these viruses, whereas only 5 per cent developed colds after a second challenge with either the same or the other virus.

Reisolation of the virus several days after challenge is another method of demonstrating infection and in vivo proliferation of virus. We succeeded in

reisolating JH virus in a high proportion of the subjects tested. JH virus was isolated on all attempts on Days, 2, 4, and 6 from the 2 subjects tested who developed symptoms of a cold; also, positive isolations were obtained from 29 per cent of specimens and from 47 per cent of subjects who did not have symptoms of a cold.

Tyrrell and Bynoe[7] recovered JH virus on the first to the fourth day after inoculation from 4 of 6 subjects who developed clinical colds and from 7 of 52 who did not develop clinical colds. Both they and we were able to transmit colds serially by using tissue culture isolates from volunteers challenged in 1 experiment to infect volunteers in a subsequent experiment.

As demonstrated by the intranasal proliferation of virus, the absence of clinical symptoms was not a perfect index of immunity to infection. It was possible to define immunity by the failure to recover virus from volunteers after challenge. Effective immunity of this type was demonstrated among volunteers who developed a cold from a previous challenge with 1 of the viruses. Prior challenge with 2060 or JH virus without a symptomatic illness was a less effective immunizing mechanism, but among such subjects the number of virus isolates was decreased after a second challenge as compared with subjects who had not been given the viruses previously.

Serologic evidence of infection as determined by the neutralization test also was observed. Subjects from whom JH virus was reisolated showed a significant rise in serum antibody for the recovered virus, which is confirmatory evidence for the occurrence of a definite infection in these volunteers. Among a larger group of volunteers challenged with either 2060 or JH, 18 per cent showed a rise in the serum neutralizing antibody titer to 2060 and 25 per cent to JH, whereas none of the volunteers who were given buffer solution showed a rise in titer against either virus. In our series, 29 per cent of 38 pairs of sera showed a rise in neutralizing antibody titer against JH after challenge with that virus.

The replicate determination of neutralizing antibody in paired sera, tested against stock virus and different batches and generations of monkey kidney cells, showed that the observed antibody titer was affected by these variables as well as by the dose of virus used in the tests, the characteristics of the maintenance media, and whether the cultures were stationary or rolled. It is clear from these observations that further work is required to standardize the technique and interpretation of tests for antibody against these viruses.

## SUMMARY AND CONCLUSIONS

One hundred and fifty-nine volunteers were challenged with either 2060 or JH viruses which had been propagated in monkey kidney tissue cultures. Ninety-six other volunteers served as controls and were challenged with uninfected tissue culture fluid harvest or a buffered saline solution. Observations were made regarding the occurrence of symptoms, recovery of virus, immunity to rechallenge with the same or other agent, and the titer and increase of neutralizing antibody in the serum.

Viruses 2060 and JH appear to be closely related. They produce similar symptoms, and, more important, immunity to one virus was demonstrated after previous challenge with the other. Immunity was shown by both decreased illness and a decrease in the proliferation of virus after rechallenge. The natural common colds observed were serologically unrelated to 2060 or JH virus. Also, subjects who had been previously challenged with an infectious filtered nasal secretion from a donor with a natural common cold did not develop immunity to challenge with 2060 or JH.

Although infections are more likely to be subclinical than to produce symptoms, it appears that viruses 2060 and JH should be accepted as infectious agents which can cause the common cold, since (1) they were isolated from persons with a respiratory infection, (2) they produce illness clinically characteristic of the common cold in an appreciable percentage of volunteers, (3) rises in the titer of neutralizing antibody have been demonstrated in the sera of subjects challenged with these viruses, (4) immunity has been demonstrated upon rechallenge with the same agents, (5) the virus has been reisolated from nasal secretions of volunteers up to 6 days after inoculation, and (6) the reisolated virus was infectious in new volunteers.

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# RHEOPLETHYSMOGRAPHIC STUDIES OF DIGITAL VENOUS TONE AND VENOUS ACTIVITY

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PREVIOUS rheoplethysmographic (RPG) studies[1-3] showed the digital veins to be active and the rheoplethysmographic method to be a means of studying aspects of the behavior of the venous side of the digital circulation of intact man. The present investigations are concerned with further studies of digital veins and the vasoconstriction (including venoconstriction) associated with obstruction to venous outflow and an increase in digital venous pressure. Data are also presented in support of the hypothesis that pre- and postcapillary vascular segments in the digit function in a manner resembling afferent and efferent arteriolar behavior of the glomerulus of the kidney. Apparently, the arteriolar or precapillary components of the circulation and the venous and venular or postcapillary components may constrict or dilate independently or simultaneously to equal or unequal amounts. This vascular function probably varies intracapillary hydrostatic pressure, rate of blood flow, and volume of different portions of the digital vascular bed, with local changes in the rates of filtration of plasmal substances into the tissues and absorption of water and other substances from the intercellular spaces.

## METHODS AND MATERIALS

The experiments were conducted in essentially the same manner previously described for other studies.[1-3] The normal subjects varied from 24 to 32 years of age and the abnormal subjects from 43 to 65 years of age. The index and third right finger tips were studied at heart level with the subjects resting in bed in an air-conditioned room (78° F. and 56 per cent relative humidity). For convenience, the details of each experiment are presented separately.

*Digital Vascular Response to Obstruction to Venous Outflow and Increase in Digital Venous Pressure.—*

*Definition of venous tone:* Venous tone is considered to represent the actively produced tension of the smooth muscle of the wall of the veins. This tension is considered to be the result of active metabolic processes within the muscle fibers associated with their resistance to distension by increased intravenous pressure. By this definition, venous tone does not represent passive resistance to distension by the smooth muscle, as would be offered by dead or nonliving material such as fibrous or elastic connective tissue or dead smooth muscle. According to the above definition, venous tone is high when the wall of a vein resists a distending force caused by high intravenous pressure by active tension. A vein could be

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of large or small diameter and the smooth muscle tone could be high or low in either instance. This capacity to vary tension, in spite of fiber length, in order to actively support through life processes high or low intravascular pressure when the vessels are engorged with blood or when nearly empty of blood, is one of the interesting physiologic attributes of the smooth muscle of the blood vessels.

The tone of the smooth muscle of a blood vessel is high when the fibers through metabolic or life processes "pull" tight around the blood column within the vessel, and tone is low when the pull or tension of the fibers is low. Thus, "vasotightening" and "vasoloosening" can occur with or without a change in luminal diameter. Tension, in this sense, differs in meaning from vasoconstriction or "vasonarrowing" and vasodilatation or "vasowidening." The vasoconstricted and vasodilated states may be associated with high or low tone of the smooth muscle, depending upon the degree of tension or pull which exists in the fibers in resistance to the force which tends to distend the vessels.

In view of the above concept of smooth muscle tone, if an experiment is designed which shows that the lumina of digital veins decrease in volume in the presence of an increased digital intravenous pressure, it must be concluded that the fibers of the smooth muscle within the digital veins shortened as a result of active physiologic phenomena and the smooth muscle tone increased. If this narrowed luminal state of the veins was maintained at a more or less fixed level for a period of time, this would be in further support of a state of increased venous tone and would distinguish increased venous tone from venoconstriction. The experiment described below will demonstrate this type of reaction.

*The experiment:* After sudden inflation of the digital collecting cuff, blood is trapped in the vessels of the digit enclosed in the plethysmographic cup and the volume of the finger tip is suddenly increased. With the collecting cuff still inflated, the digital volume still increased, and the occluding pressure maintained, if the digit were to decrease in volume by an amount varying from a little to more than 100 per cent of the volume of blood originally trapped in the finger tip immediately after inflation of the collecting cuff, digital vascular tone must have increased. This was found to occur (Fig. 1, and Table 1). With sudden release of the pressure within the collecting cuff, there was a sudden decrease in volume of the digit, which usually resulted in an overshoot of the volume trace beyond the original level of the volume base line (Fig. 1). This sudden after-drop, primarily an artifact,[4, 5] was usually followed by a gradual return of the volume trace to the original digital volume base line. This entire response was observed in this laboratory over 20 years ago,[6] as well as by other investigators.[4, 5, 7] The experiment was repeated at several levels of collecting pressure and many times for the same subject (Table 1).

Curves of time courses of digital inflow ($I_V$ and $I_R$) and outflow ($O_V$ and $O_R$) for the first pulse cycle immediately after inflation of the collecting cuff, and for the third pulse cycle immediately after the after-drop are shown in Fig. 2, *a* and *b*. There was a decrease in the volumes and rates of digital inflow and outflow following the after-drop. In other experiments the magnitude of decrease was variable but never large.

That digital venous tone increased after obstruction to venous return is supported by the following data (Figs. 1 and 2): with sudden inflation of the collecting cuff, digital venous flow was interrupted and inflowing blood was trapped within the digital vessels, as indicated by the increase in total volume of the digit in the RPG. The digital vascular walls were distended and the intraluminal pressure was increased. The A-V pressure gradient gradually decreased as the pressure in the veins distal to the collecting cuff rose, until the venous pressure exceeded the pressure in the collecting cuff, whereupon outflow developed and gradually increased until it equaled inflow. When this occurred the curve of total digital inflow volume of the RPG became essentially horizontal. When the pressure in the collecting cuff was maintained for several minutes, the volume of the digit distal to the collecting cuff decreased. At times, the total decrease in volume exceeded the increase in volume which occurred immediately after inflation of the collecting cuff (Table 1). It is generally believed, and, apparently, correctly so, that the inflowing blood collected distal to the collecting cuff accumulated in large part within the veins and venules of the digit. Therefore, it would

TABLE I. DIGITAL VOLUME CHANGES IN SELECTED RHEOPLETHYSMOGRAMS

| SUBJECT NO. | COLLECTING PRESS (MM. HG) | TOTAL VOLUME INFLOW (C. MM./5 ML. PART) | VOLUME DECREASE DURING OCCLUSION: SPONTANEOUS (C. MM./5 ML. PART) | VOLUME DECREASE DURING OCCLUSION: RESPONSE TO BELL (C. MM./5 ML. PART) | VOLUME DECREASE DURING OCCLUSION: SPONTANEOUS (%) | VOLUME DECREASE DURING OCCLUSION: RESPONSE TO BELL (%) | VOLUME DECREASE ON RELEASE OF CUFF*: (C. MM./5 ML. PART) | VOLUME DECREASE ON RELEASE OF CUFF*: (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | 20 | 7.2 | 6.0 | | 83 | | 18.5 | 161 |
| | 30 | 20.8 | 26.4 | | 127 | | 31.5 | 107 |
| | 40 | 40.3 | 51.7 | | 128 | | 35.1 | 63 |
| | 50 | 37.2 | 54.8 | | 147 | | 50.0 | 90 |
| | 60 | 45.6 | 32.2 | 10.1 | 71 | 22 | 62.6 | 98 |
| | 70 | 48.4 | 45.9 | | 95 | | 80.6 | 106 |
| | 80 | 59.8 | 67.2 | | 112 | | 115.7 | 129 |
| | 90 | 37.2 | 49.6 | | 133 | | 98.1 | 140 |
| 1 | 20 | 9.5 | 0.0 | 26.8 | 0 | 282 | 15.4 | 102 |
| | 40 | 13.1 | 1.2 | 13.1 | 9 | 100 | 30.6 | 79 |
| | 60 | 50.0 | 0.6 | 14.9 | 1 | 30 | 63.6 | 103 |
| | 60 | 47.0 | 0.0 | 19.0 | 0 | 40 | | |
| | 60 | 47.6 | 0.0 | 9.9 | 0 | 19 | | |
| | 80 | 43.4 | 18.6 | 9.8 | 43 | 23 | 63.0 | 100 |
| 3 | 20 | 0.0 | | | | | | |
| | 30 | 4.7 | 6.3 | 34.4 | 134 | 732 | 25.4 | 90 |
| | 40 | 17.4 | 16.8 | 15.3 | 97 | 88 | 38.3 | 88 |
| | 50 | 27.3 | 17.2 | 26.5 | 63 | 97 | 53.1 | 89 |
| | 60 | 16.4 | 46.5 | 21.3 | 284 | 130 | 61.2 | 100 |
| | 60 | 28.9 | 17.9 | 24.6 | 62 | 85 | 59.2 | 84 |
| | 70 | 35.6 | 15.9 | 18.2 | 45 | 51 | 83.9 | 99 |
| | 80 | 39.0 | 16.7 | 19.3 | 43 | 49 | 79.8 | 92 |
| | 90 | 38.8 | 20.8 | 35.9 | 54 | 93 | 59.8 | 65 |
| | 100 | 44.8 | 10.9 | 21.3 | 24 | 48 | 87.4 | 89 |
| 8 | 20 | 0.0 | | | | | | |
| | 30 | 20.4 | 41.1 | | 201 | | 21.7 | 84 |
| | 40 | 53.2 | 37.2 | | 70 | | 44.3 | 76 |
| | 50 | 71.7 | 48.5 | | 68 | | 49.9 | 66 |
| | 60 | 71.7 | 73.9 | | 103 | | 66.1 | 87 |
| 9 | 20 | | | | | | | |
| | 30 | 7.7 | 13.8 | | 179 | | 27.1 | 169 |
| | 40 | 27.5 | 16.3 | | 59 | | 58.1 | 150 |
| | 50 | 48.8 | 21.8 | | 45 | | 71.9 | 107 |
| | 60 | 65.1 | 54.4 | | 84 | | | |
| 10 | 20 | | | | | | | |
| | 30 | 26.7 | 7.6 | | 28 | | | |
| | 40 | 49.9 | 26.1 | | 52 | | 46.4 | 79 |
| | 50 | 51.0 | 34.2 | | 67 | | 73.4 | 110 |
| | 60 | 65.5 | 19.7 | | 30 | | 82.6 | 100 |
| 11 | 20 | 27.2 | 3.0 | | 11 | | | |
| | 30 | 23.0 | 57.2 | | 249 | | 59.3 | 159 |
| | 40 | 52.4 | 24.2 | | 57 | | 76.0 | 141 |
| | 50 | 56.9 | 29.1 | | 51 | | 53.4 | 73 |
| | 60 | 56.0 | 47.5 | | 85 | | 50.6 | 71 |
| 12 | 60 | 23.8 | 4.3 | 30.1 | 18 | 126 | 63.8 | 174 |
| | 60 | 36.5 | 9.7 | 6.8 | 27 | 19 | 44.0 | 92 |
| | 80 | 37.8 | 9.2 | 12.0 | 24 | 32 | 80.5 | 113 |
| | 80 | 47.5 | 3.9 | 9.2 | 8 | 19 | 72.8 | 88 |
| 13 | 20 | 3.4 | 17.1 | | 503 | | 13.3 | 177 |
| | 30 | | | | | | 27.7 | 129 |
| | 40 | 20.6 | 22.0 | | 107 | | 32.2 | 81 |
| | 50 | 36.3 | 13.7 | | 38 | | 56.3 | 91 |
| | 60 | 48.0 | 30.2 | | 63 | | 69.3 | 94 |

*Not corrected for artifact.

also be expected that, if the volume of the engorged finger decreased in an amount equal to, or nearly equal to, the original volume of engorgement, part of the decrease in volume must have occurred in the digital veins. Furthermore, whenever venous volume decreased, regardless of how infinitesimal the decrease, while the collecting cuff was inflated, the smooth muscle in the walls of the veins contracted and the tension developed, exceeding that which existed previously, that is, venoconstriction occurred. Fig. 2 indicates that the precapillary vessels alone could not account for the total decrease in digital volume, because these vessels could not have constricted very much, as indicated by the relatively small decrease in the rate of digital inflow measured immediately after release of the collecting cuff. It is also unlikely that decrease in volume of the capillaries alone could account for the magnitude of volume change observed in some experiments. Therefore, the veins must have constricted and contributed to the decrease in digital volume.

At times, the decrease in volume of the finger tip which occurred while the collecting cuff was still inflated must have been stimulated in part by an "orienting reflex" induced through the sense of touch by squeezing of the digit by the collecting cuff.[2] The decrease in volume was partly arteriolar and/or arterial in origin since the volume and rate of inflow decreased and the volume of the pulse wave was reduced. However, vasoconstriction, alone,

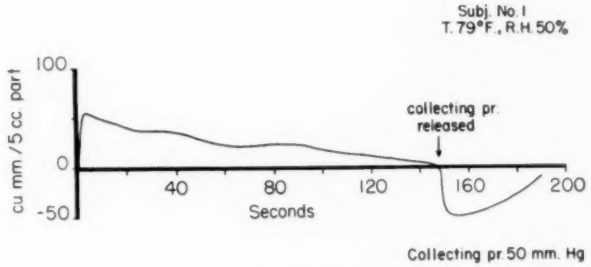


Fig. 1.—Digital vasoconstriction with venous obstruction (2RF). Time course of the total digital volume for the right index finger tip (2RF) simplified from the original rheoplethysmogram. The immediate increase in digital volume after inflation of the collecting cuff was followed by a spontaneous decrease in finger volume while the collecting cuff was still inflated and digital venous pressure elevated. The after-drop upon release of collecting cuff pressure is shown.

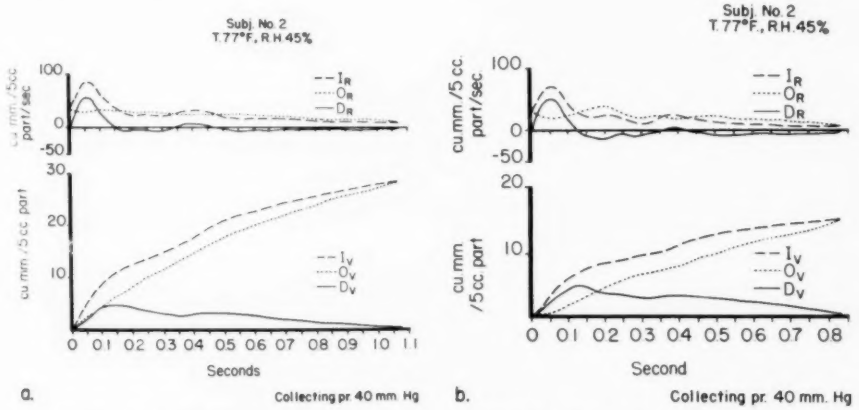


Fig. 2.—The digital rheoplethysmogram (control 2RF) for the right index finger tip at time of inflation of the collecting cuff is shown in *a* (digital rheoplethysmogram 2RF) the ntrol record, and that for the third pulse cycle after deflation of the collecting cuff and lease of venous obstruction is shown in *b*. The rates of inflow, $I_R$, and outflow, $O_R$, and lume of inflow, Iv, and outflow, Ov, were decreased after prolonged inflation of the collecting ff. The $D_R$ and $D_V$ curves are curves of the differences between the rates and volumes of low and outflow, respectively. The symbols employed in this illustration are used throughout is report and in rheoplethysmography.

produced by the orienting reflex, was not sufficient to account for the volume change noted and the volume change did not display the time course of the response to an orienting reflex. The vasoconstrictor response to the orienting reflex is characterized by a rather sudden constriction of the digital vascular bed (beginning about 2.5 seconds after initiation of the reflex) with a relatively rapid return of the vascular system to its original volume. The major digital vasoconstriction and increase in smooth muscle tone in response to inflation of the collecting cuff developed slowly, were usually stepwise, were often of considerable magnitude, and lasted for many minutes if entirely released. The RPG patterns of these two vasoconstrictor responses are quite different.[2]

The increase in digital vascular tone in response to inflation of the collecting cuff is diffusely distributed among the vessels of the digit, and the magnitude of the respective vascular segmental contributions remains unknown. The reaction is not entirely passive. The exact location of the receptor and effector organs and the mechanism of the reactions are also unknown.

There was a limit to the pressure which venous contraction would overcome,[1] although the volume of the vessels of the digit decreased to some extent against intravascular pressures exceeding 100 mm. Hg (Table 1). The degree of muscle tone or fiber tension developed can be estimated from the level of pressure in the collecting cuff.

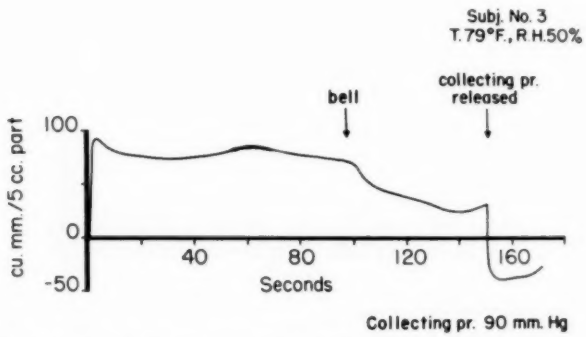


Fig. 3.—Time course of total digital volumes during inflation of the collecting cuff, showing vasoconstriction, which most likely included venoconstriction, in response to the sound of a bell (2RF). Graph was simplified from the original recording.

To show that the digital vascular bed can constrict against a pressure of 90 mm. Hg or more, a bell was rung while the collecting cuff was inflated and the digital vessels distended under increased pressure. The stimulus initiated by the sound resulted in digital vasoconstriction (Fig. 3). No attempt was made to determine the portions of the digital vascular bed involved, but previous studies showed that the response involved both the arterial and venous sides of the circulation. This type of response is similar to that obtained by Capps[3] after the skin was pinched.

The after-drop, discussed in detail by Gaskell[4] and Allwood,[5] is primarily an artifact. Excessive significance was placed upon it initially by Gaskell and Burton.[7] However, it probably does reflect, to some degree, an overshoot in volume of the digital vessels caused by increase in vascular tone, probably venous in large part, which becomes manifested in the after-drop associated with sudden release of venous obstruction by sudden deflation of the collecting cuff. The slow return of the digit to its original volume with refilling of the digital vascular bed is probably, in part, an expression of the return of arterial and venous tone to that originally present prior to inflation of the collecting cuff. Were the refilling of the digit after the overshoot merely passive and not a readjustment of vascular tone, the vessels would refill to the previous resting volume more rapidly in view of the relatively rapid rates of inflow recorded for the digit at the time of the after-drop (Fig. 2, *a* and *b*). Furthermore, blood was found to be flowing out of the digit while the volume curve, after

the overshoot, was still rising ($O_V$ and $O_R$ of Fig. 2, *b*). Such a high rate of digital outflow would not likely occur if there were a need for passive refilling of the digital vessels. It is reasonable, however, to expect the sudden emptying of some of the blood from the digit to redistend the veins which were partially compressed by the collecting cuff to contribute in part to the after-drop. The studies of Gaskell[4] and Allwood[5] support this explanation, but do not eliminate all other possible contributing factors to the volume-time course curve of the after-drop.

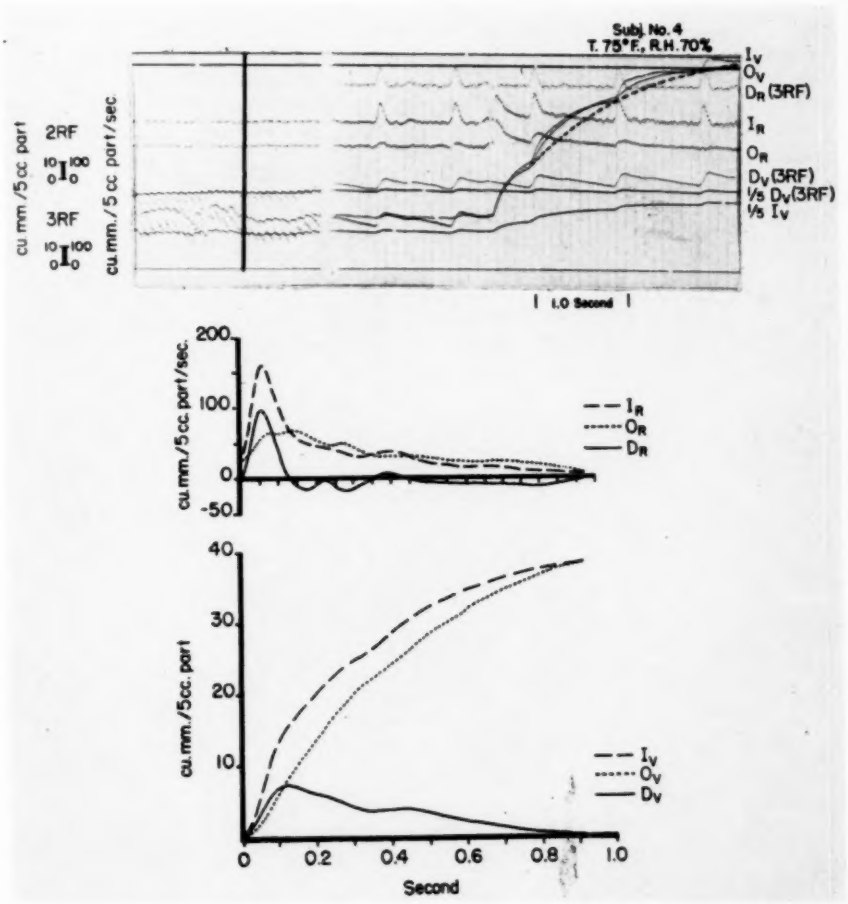


Fig. 4.—The original normal (2RF) rheoplethysmogram (RPG) and the rate- and volume-time course curves for a single pulse cycle, with the subject comfortably at rest in a neutral environment. This RPG is typical of a moderately relaxed digital vascular system. The dotted line in the original RPG shows the gradual decrease in the slope of the volume curve of inflow. Consult the text for details.

*Time Course of Digital Volume Immediately After Inflation of the Collecting Cuff.*—It had been noted for some time that the configurations of the time course curves of total digital volume after sudden inflation of the collecting cuff were essentially of 4 major types:

1. *The volume-time course curve associated with a moderately relaxed digital vascular system (Fig. 4):* This type of curve is characterized by a fairly rapid increase in volume of the digit after sudden inflation of the collecting cuff, with an early and gradual decline in the rate of increase in digital volume as evidenced by the curve approaching the

horizontal asymptotically (dotted line in original record of Fig. 4). This type of curve was associated with a relaxed venous system, that is, one of moderate degree of venous distention and tone. The veins and other collecting vessels of the digit were able to accommodate fairly large volumes of blood before the A-V pressure gradient fell markedly.

2. *The volume-time course curve associated with a greatly dilated digital vascular system (Fig. 5):* This volume-time course curve is characterized by a rapid rise after sudden inflation of the collecting cuff with rapid attainment of a horizontal course or plateau. This type of RPG was found for a subject exposed to a hot and humid atmosphere. The curve apparently developed because of several factors: (1) an almost maximally dilated digital vascular system engorged with blood prior to inflation of the collecting cuff caused by dilatation from the heat and humidity, (2) a rapid rate of digital inflow so that maximal distention of the vascular bed with blood is reached within a small fraction of one pulse beat,

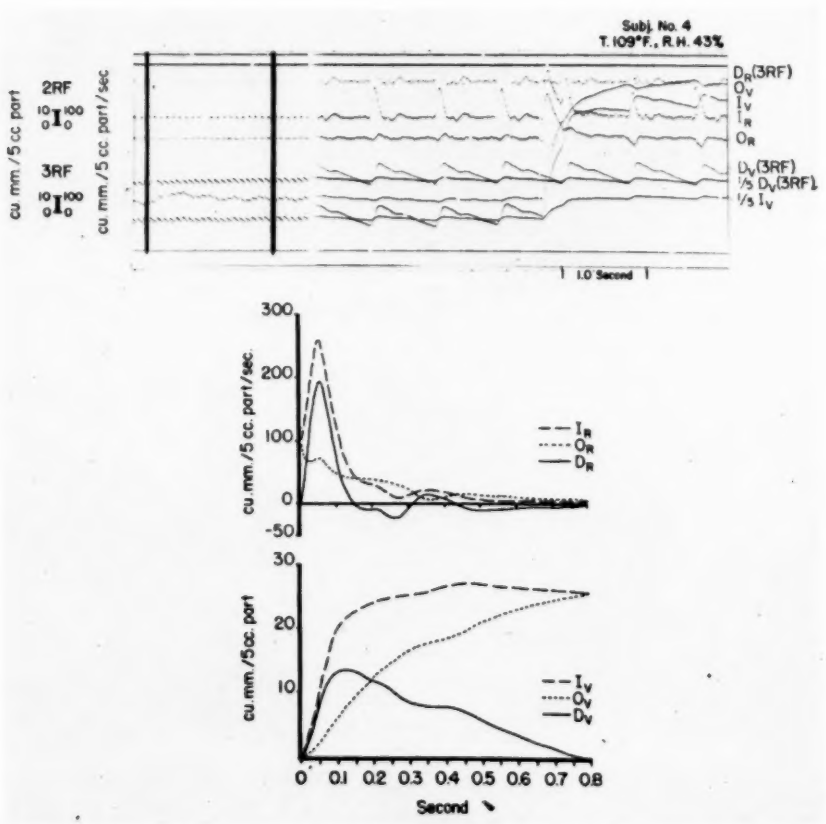


Fig. 5.—The original normal digital RPG (2RF) and the rate- and volume-time course curves for a single pulse cycle with marked digital vasodilatation produced by a hot and humid environment. The curve of volume inflow rose suddenly and reached a slope of essentially zero within the interval of the first pulse cycle.

(3) a rapid decline of the A-V pressure gradient to its minimal level so that the rate of digital inflow declines quickly, and (4) the pressure in the veins distal to the collecting cuff rapidly exceeding the pressure in the collecting cuff so that the large quantities of inflowing blood escape past the collecting cuff and do not remain in the digit to be measured. This type of curve has been described in detail previously.[2]

When such volume curves were encountered, the rate of basal pulsatile flow, i.e., rate of flow at $t_a$ or onset of systole,[2] was high because of the dilated arterioles, small arteries, and A-V shunts (Fig. 5). The rate of basal flow is one of the most reliable indices of the degree of dilatation or constriction of the arterioles, arteries, and A-V shunts. All aspects of the RPG must be integrated for proper interpretation of the trace in terms of the state of the peripheral blood vessels.

3. *The volume-time course curve of the constricted digital vascular system:* This curve was of 2 types, depending upon whether or not there was an increase in digital volume during the diastolic phase of the pulse cycle. In both types of curves there was an increase in volume during the systolic phase of the pulse cycle. Both types of traces were associated with a constricted vascular system. These curves showed relatively small increase in volume

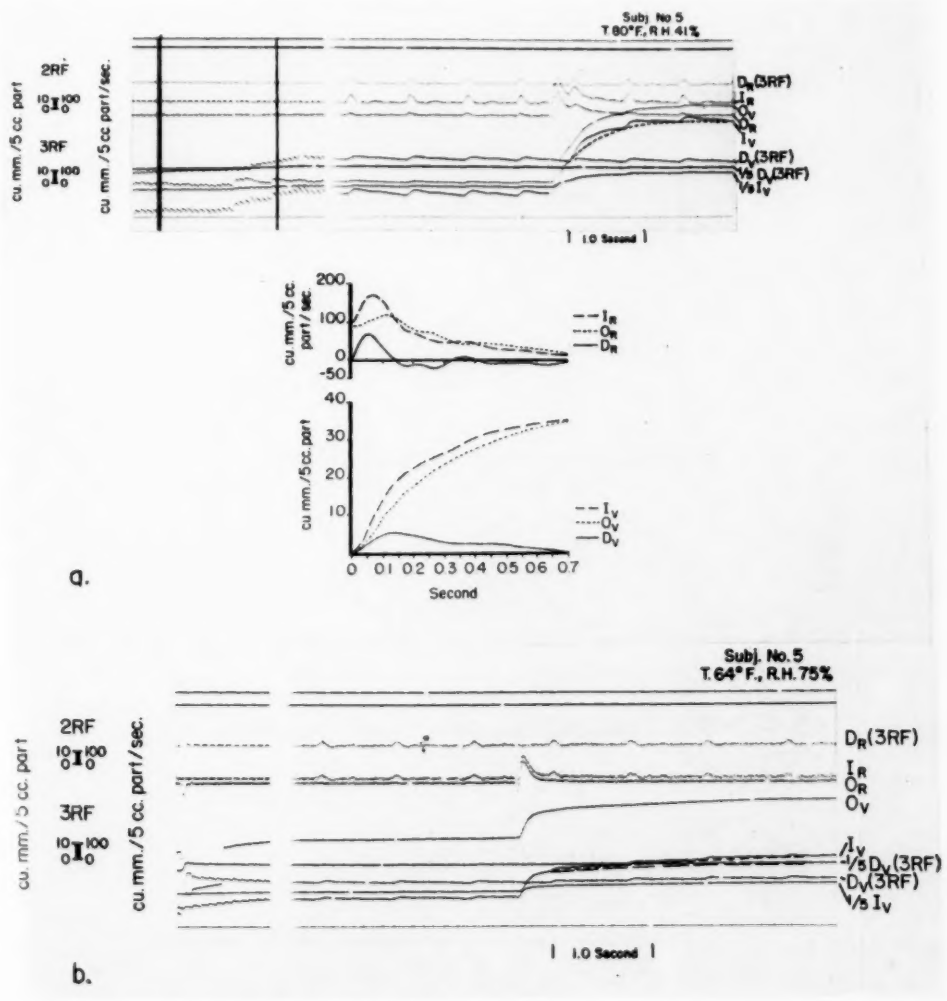


Fig. 6.—The original normal digital RPG and the rate- and volume-time course curves for a single pulse cycle for Subject 5 in a neutral (*a*) and in a cool (*b*) environment. The interrupted line in *b*, composed of short dashes, shows the constant increase in volume of the digit over a period of several pulse cycles for the subject in a cool environment. The other interrupted line composed of long dashes is the artifact volume curve. From these 2 interrupted lines it is evident that the digit increased in volume only during systole.

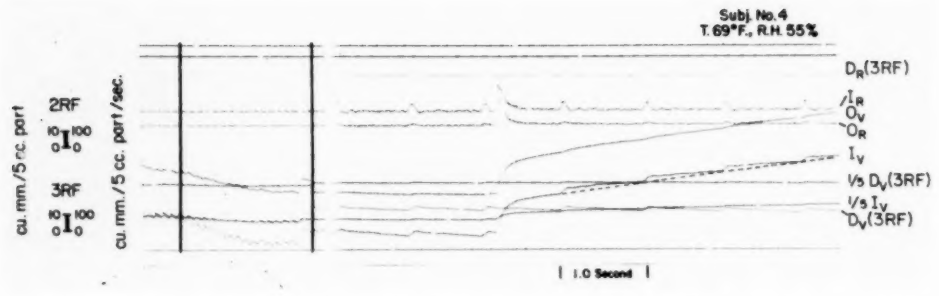


Fig. 7.—The original normal digital RPG (cold environment 2RF) showing a constant mean rate of increase in digital volume over several pulse cycles with an increase during both the systolic and the diastolic phases of the pulse cycle.

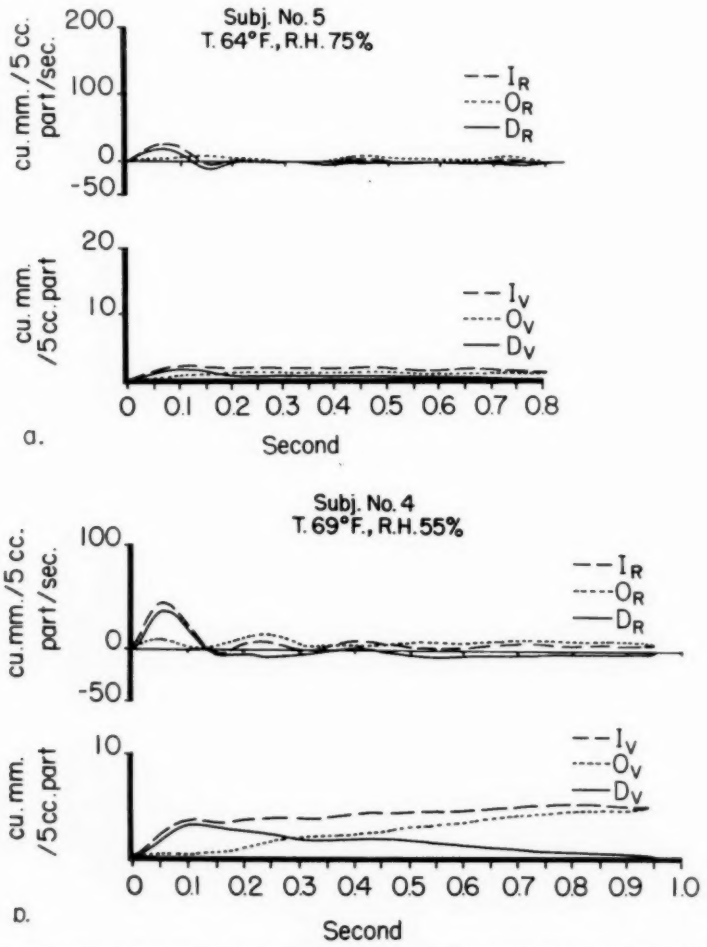


Fig. 8.—The rate and volume curves for a single pulse cycle for the normal RPG (cold environment 2RF) shown in Figs. 6, *b* and 7, respectively. These curves show the absence of digital flow during the diastolic phase of the pulse cycle for the first group of curves (Subject 5) and the presence of flow during diastole for the second group (Subject 4).

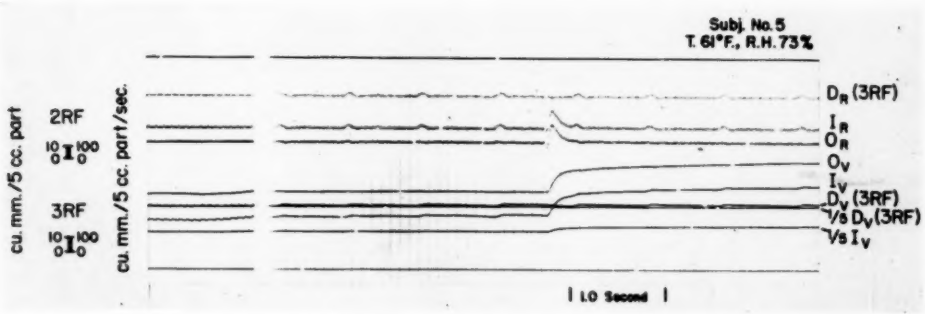


Fig. 9.—The original normal digital RPG of Subject 5 when extremely cold (2RF), showing no measurable digital blood flow.

after inflation of the collecting cuff (Figs. 6 and 7). Both volume curves rose at a relatively constant rate for many pulse cycles before they began to show a decline in the rate of rise, a distinct difference from the curves associated with a relaxed or a markedly dilated digital vascular system (Figs. 4-7). One type of volume curve (Type A) rose little, if at all, or even declined during the diastolic period of the pulse cycle (Fig. 6, *b*), whereas the other (Type B) rose continuously during the diastolic, as well as during the systolic, phase of the pulse cycle (Fig. 7).

These 2 types of volume-time course curves require special comment. The curves have been encountered only during periods of digital vasoconstriction and states of relatively high digital vascular tone. They were collected by making periodic RPG recordings while the air in the subject's room was slowly cooled. As vasoconstriction progressed, the 2 patterns shown in Figs. 6, *b* and 7 gradually developed. These volume curves can also be produced by means of psychic or sensory stimuli.

The Type A volume curve shown in Fig. 6, *b* is characterized by low digital inflow with essentially no inflow during diastole. Inflow, therefore, occurred only during systole when arterial pressure was highest. The high intravascular pressure during the systolic phase of each succeeding pulse cycle apparently distended the vascular bed which retained each newly attained volume of blood. There was little or no further accumulation of blood in the digit during the diastolic phase of the pulse cycle because of either (1) lack of flow into the digit during the diastolic period caused by the low arterial pressure, or (2) equality of the rates of inflow and outflow during diastole. However, the existence of inflow of blood during diastole was occasionally established by inflation of the collecting cuff during the diastolic phase of the pulse cycle. Digital inflow, though small, was present. Therefore, the absence of recorded blood flow during the diastolic phase of each succeeding pulse cycle for the type of curve shown in Fig. 6, *b* is the result of a decline in the A-V pressure gradient after the first pulse beat, with rate of leakage past the collecting cuff during the diastolic phase of the pulse cycle equal to the rate of inflow, so that there is no net change in digital volume.

The arterial and arteriolar constriction produced by the cold resulted in a low A-V pressure gradient during diastole and a "tight" venous reservoir or "tight" collecting system. The constricted collecting vessels could not distend further to accommodate the small amount of inflowing blood when subjected to the relatively low pressure during diastole. The pressure in the veins, therefore, rose rapidly to exceed that in the collecting cuff so that the rate of leakage past the collecting cuff equalled the rate of inflow.

The experiments apparently justify the following conclusions: with cooling of the subject, vasoconstriction of the entire digital vascular system developed, the rate of digital inflow decreased, and, although venous constriction was associated with an increase in venous tone, the pressure and force during the systolic phase of the pulse cycle were sufficiently high

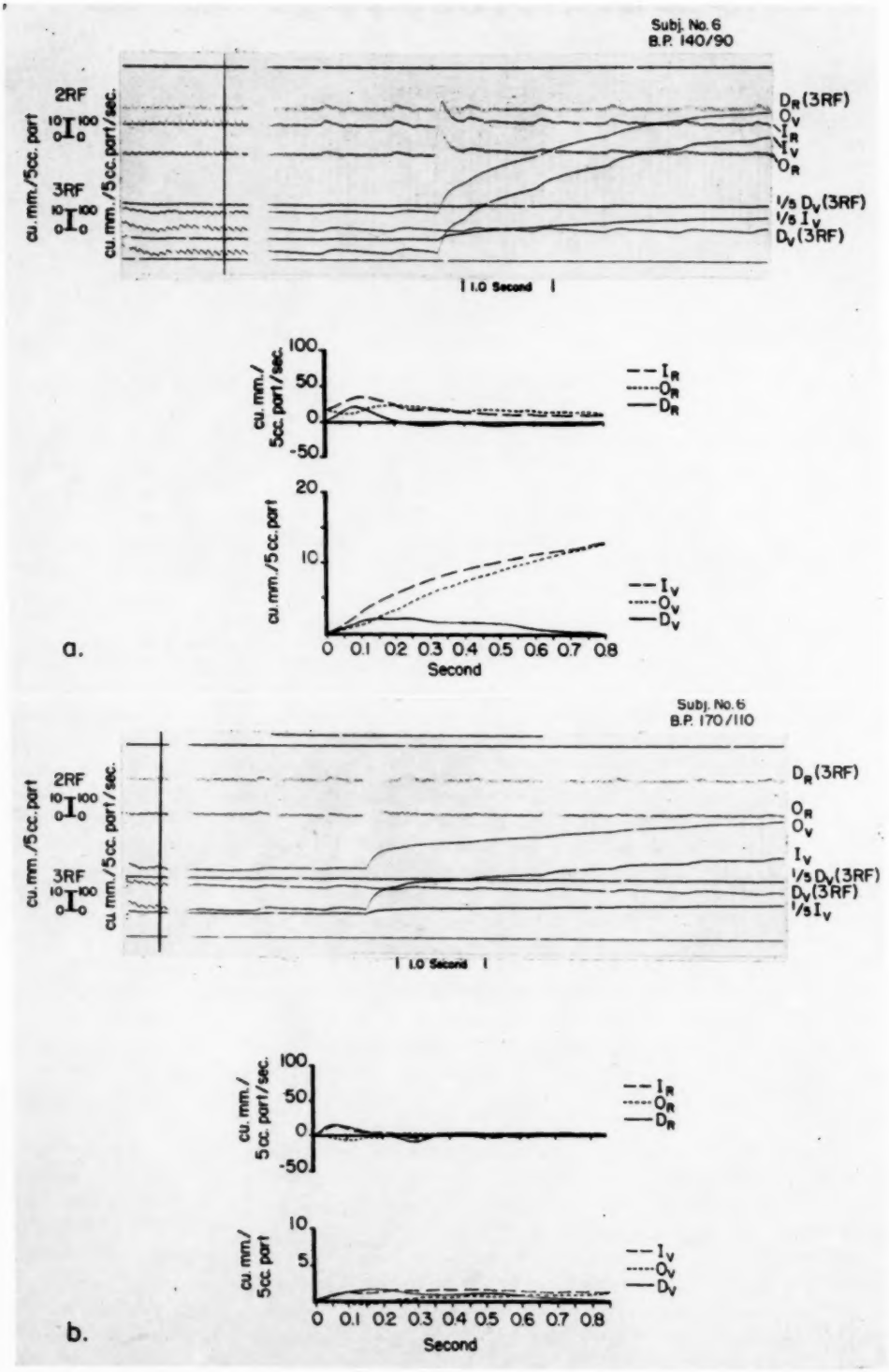


Fig. 10.—The digital RPG before (*a*) and after (*b*) intravenous administration of noradrenalin. 2RF. Consult the text for details.

to stretch the digital vascular collecting system with a resultant net gain in volume of the digit. Once stretched with blood, the vessels of the digit retained the increased volume of blood but accumulated little or no further blood during diastole. This process repeated itself quantitatively with each successive pulse beat for a relatively long period of time to produce a uniform rate of increase in digital volume.

The Type B volume curve probably develops for the same reasons as those discussed for Type A, with the exception that, for the former type, the forces in the vessels during diastole were sufficiently great to distend the collecting vessels, including the digital veins. Thus the collecting vessels were able to accommodate the inflowing blood with little or no leakage past the collecting cuff or with the rate of leakage less than the rate of inflow. It would appear, therefore, that venous tone, high for both types of curves, was less when the Type B curves were obtained than when Type A curves were obtained. The $R_V$ and $R_R$ families of curves (Fig. 8, *a* and *b*) indicate greater arterial and arteriolar constrictor response to the cool atmosphere for Type A than for Type B curves. These types of response

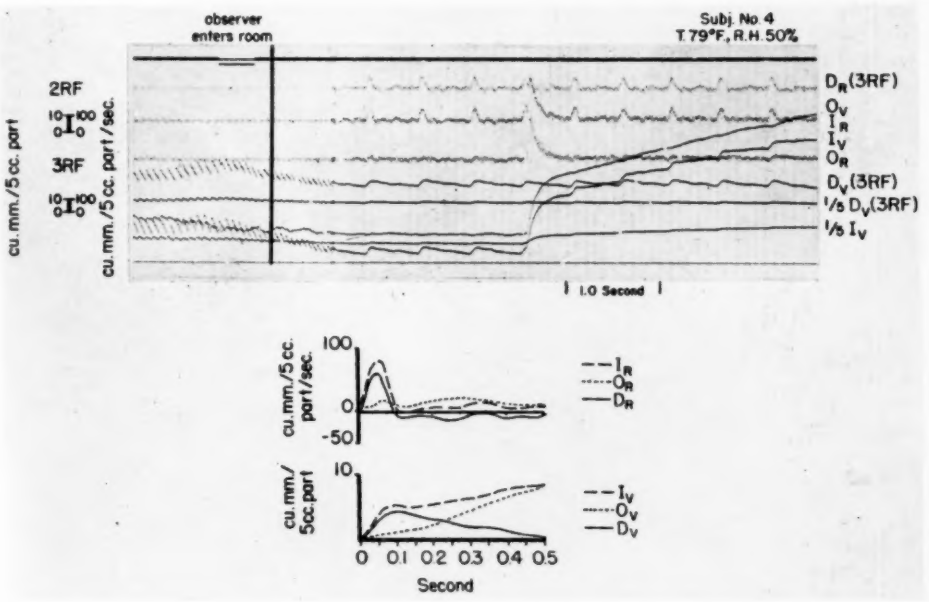


Fig. 11.—The normal digital RPG showing the influence of the orienting reflex (2RF) initiated by an observer entering the room with the patient. The digital vasoconstriction is evident in the slow speed recording. The original RPG and rate and volume curves recorded during this period of vasoconstriction can be compared with those in Fig. 4 for the neutral resting or relaxed state of the digital circulation of the same subject.

are also significant because they support the concept of disproportionate constriction of the pre- and postcapillary vessels, a vascular phenomenon of possible importance in the regulation of fluid and solute exchange across the capillary and venular walls.

During the period of cooling and when the vasoconstrictive patterns were obtained, the rates of digital flow were so low that the large arteriovenous anastomotic channels, including the glomus bodies, must have been completely or almost completely closed.[9-13] The low rate of the basal component of pulsatile flow indicates most reliably the state of marked constriction of arteries, arterioles, and A-V shunts.

4. *The volume-time course curve associated with arterial obstruction:* Volume-time course curves characteristic of arterial obstruction are produced by complete or almost complete obstruction to digital flow caused by either organic disease or marked constriction of the precapillary vessels. When the subject rested in the cool room atmosphere (50 to 54° F.),

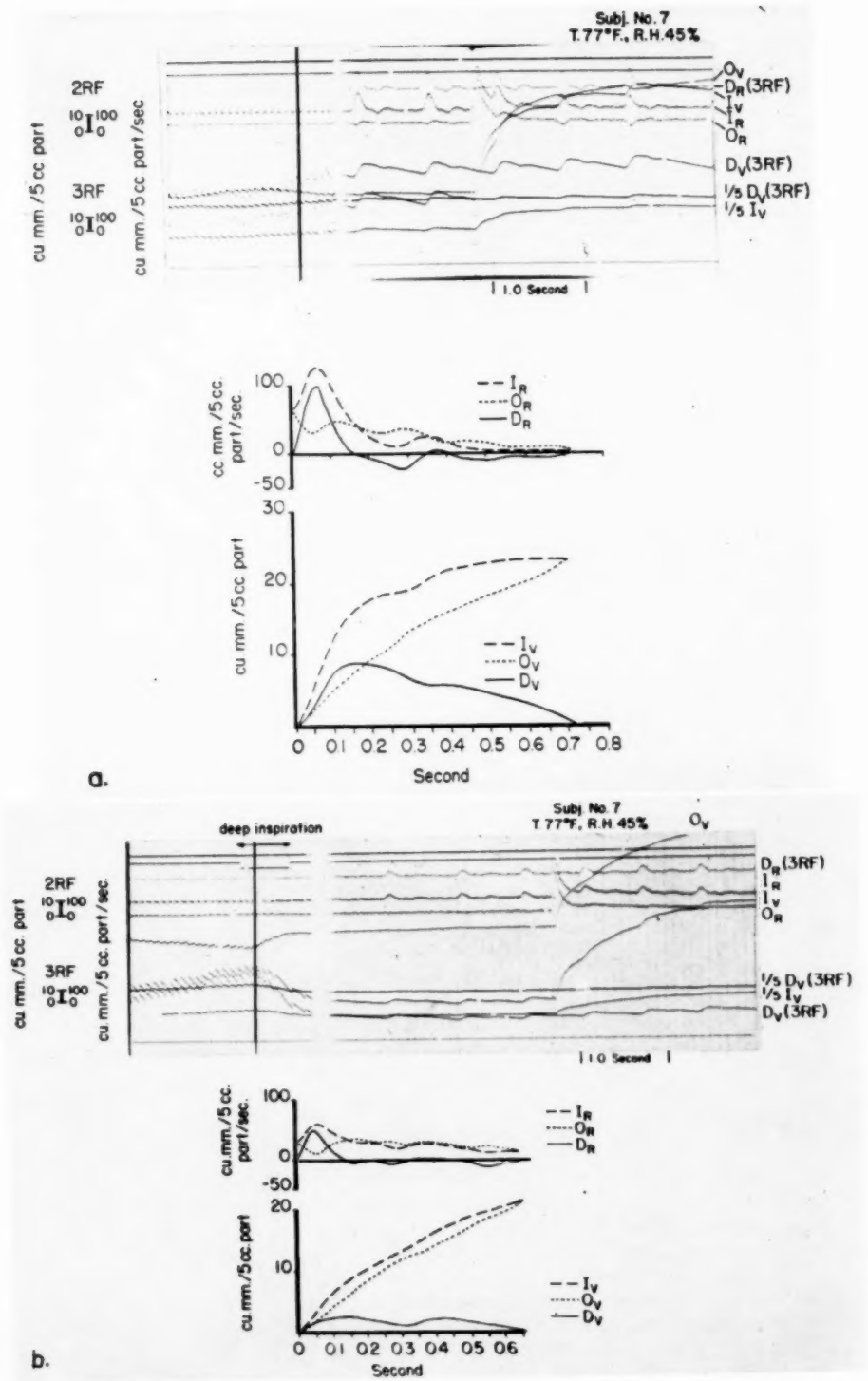


Fig. 12.—The normal digital RPG (2RF) before (*a*), and after (*b*) vasoconstriction produced by deep inspiration in Subject 7.

with only the pelvic region covered with a sheet, digital vasoconstriction became so intense as the subject cooled that no measurable digital inflow was recorded (Fig. 9), indicating closure of all A-V anastomotic channels.

The marked decrease in total volume of the digits as cooling progressed indicated an increase in venous tone and venoconstriction, the decrease in volume being too large to be caused entirely by precapillary vascular constriction. Furthermore, with cooling, the veins of the arms of the subjects were also grossly constricted. Venous pressure was found to be increased in the superficial veins of the arms of subjects exposed to a cool atmosphere.[14] Under such circumstances digital venous pressure must also be elevated.[15] The rate of basal pulsatile flow was too small to measure, further evidence of marked constriction of the precapillary vessels and A-V shunts.

*Effects of Noradrenalin on the Digital Veins.*—Noradrenalin is a strong constrictor of arteries, arterioles, and veins.[15, 16] Its action upon small veins and venules, such as those in the digits, has received little attention. During a study of the activity of the postcapillary digital vessels, noradrenalin was administered intravenously in 5 per cent glucose in water (4 mg. per 500 ml.) to normal adults while the digital rheoplethysmogram was recorded. The subjects were at rest in bed in a comfortable room atmosphere with the digits at heart level. The noradrenalin dripped into the median basilic vein in the arm opposite to that under study. The dose was regulated to produce a definite rise in arterial blood pressure, after which the infusion was stopped; recordings were obtained during the periods of increasing and decreasing arterial blood pressure (Fig. 10, *a* and *b*).

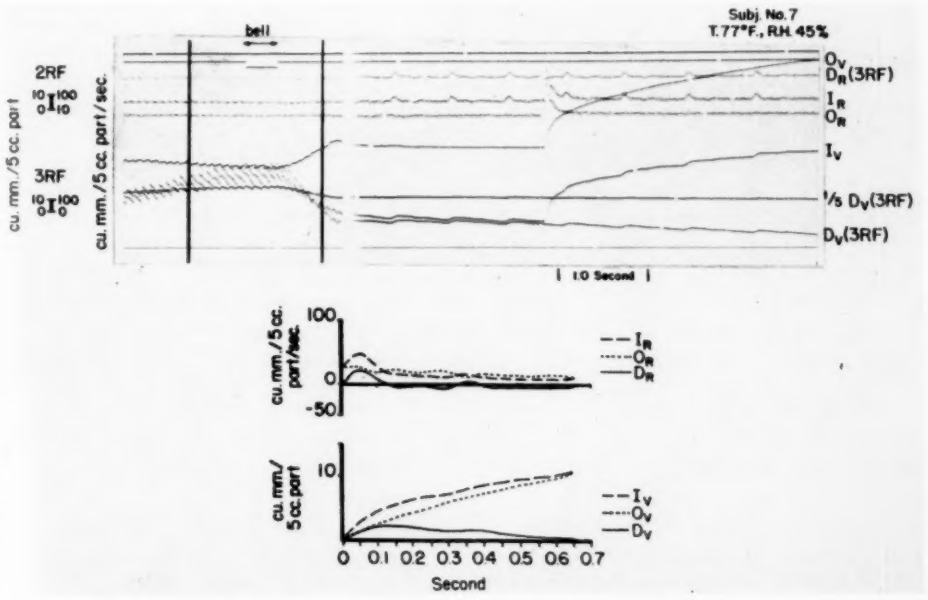


Fig. 13.—The normal digital RPG of Subject 7 showing the vasoconstriction produced by the sound of a bell, 2RF. Fig. 12, *a* shows the RPG for the neutral relaxed state.

The rates of digital inflow and outflow decreased under the influence of the dose of noradrenalin used (Fig. 10, *a* and *b*). Although the veins constricted and venous tone increased, the constriction of the precapillary vessels was greater than that of the postcapillary vessels. The relatively small increase in postcapillary vascular tone was supported by failure of the collecting reservoirs to overdistend and venous pressure to rise rapidly to levels exceeding that in the collecting cuff (Fig. 10, *b*) at the same time that total digital volume

decreased.[15] The existence of practically zero outflow during the initial phase of the pulse cycle[15] suggests high tone and constriction of the large A-V shunts. The possible physiologic significance of this type of RPG has been discussed.[17] The magnitude of the decrease in total volume of the finger tip was compatible with an associated decline in volume of the venous side of the circulation, although, with the doses used, the decrease was not as marked as the decrease encountered when the subject was cooled.

Thus, noradrenalin, in the doses administered, had a greater influence upon the precapillary vessels or afferent vessels and the large A-V shunts than upon the postcapillary vessels or efferent vessels; however, it produced constriction of all vessels. This phenomenon is analogous to the disproportionate degrees of constriction of the afferent and efferent arterioles or pre- and postcapillary vessels of the renal glomerulus. The rheoplethysmograms also showed that noradrenalin produced constriction of larger digital veins.

*Effects of Orienting Reflex, Psychic Factors, and Deep Inspiration on Digital Venous Tone.*—When a stimulus was applied directly to the subject or introduced into his environment, causing him to "orient" or to "reorient" himself within his surroundings and to become aware of the change, a digital vasoconstriction occurred in association with the orienting response. A typical response recorded rheoplethysmographically is shown in Fig. 11. It consisted of a decrease in total digital volume and decrease in volumes and rates of digital inflow and outflow (Figs. 4 and 11). The response became progressively less intense and finally failed to develop with frequent repetition of the stimulus at short intervals of time. The response to the orienting reflex varied in degree and ease of production among subjects and from time to time for the same subject; it was best produced when the tone of the digital vascular system was in a relaxed or resting state, and most difficult to elicit, if at all, when the digital vessels were under the influence of stimuli which produced marked constriction or marked dilatation.

That the digital arteries, arterioles, and A-V anastomotic channels constricted and their lumina decreased in size in response to the orienting reflex was evident from the rheoplethysmograms. For example, the total volume of the digit decreased, the volume of the pulse wave, $D_V$, decreased, and the volumes and rates of inflow, $I_V$ and $I_R$, and the basal component of pulsatile flow also decreased (Figs. 4 and 11). Such responses are characteristic of constriction of arteries, arterioles, and A-V shunts.

The orienting reflex also produced digital venoconstriction and an increase in venous tone. This response was supported by the decrease in total volume of the digit, which was great enough, 40 to 50 c. mm. per 5 ml. of part at times, to indicate constriction of the veins.

The same types of digital vascular responses and RPG patterns were obtained with psychogenically initiated disturbances, including stimuli induced by spontaneous thought. Attempts at solution of a simple arithmetic problem (multiplication or addition), conversation, fright, and the like resulted in digital vasoconstriction which involved the arterial and venous sides of the circulation. Deep inspiration also produced the same type of digital vascular response in which digital venous tone as well as arteriolar tone increased (Fig. 12, *a* and *b*).

*Sensory Stimuli and Digital Venous Tone.*—The application of stimuli, such as flashing a light, ringing a bell, pricking the subject with a pin, pinching the skin, applying heat or cold to a local area of skin, or creating loud noises, produced the same type of response in the digital vascular system (Fig. 13) as described above for the orienting reflex. Total digital volume decreased markedly and the magnitude of $I_V$, $I_R$, $O_V$, and $O_R$ declined (Fig. 12, *a* and 13). The collecting vessels became tighter or of higher tone as indicated by the marked decrease in the total volume of the digit, 50 c. mm. per 5 ml. of part, or more in some instances.

From the nature of these responses it is evident that many different sensory and psychic stimuli other than those mentioned above, some tried and some untried, would evoke the same type of response in which the digital veins would participate in association with the precapillary vessels.

*Evidence for Independent Pre- and Postcapillary Vascular Constriction in the Digital Vascular System Hypotheses.*—It is postulated that small precapillary vessels, such as the arterioles, can constrict and reduce the rate of flow and intravascular pressure in the capillaries and other digital vessels, independent of equal active constriction of postcapillary vessels. There may also be independent or disproportionate constriction of the postcapillary vessels, such as the venules and small veins, which would increase intravascular pressure in the small vessels proximally, and thus increase the rate of capillary filtration. The rate of blood flow would tend to be reduced, depending upon the degree of constriction of the pre- and/or postcapillary vessels. It is also possible to visualize dilatation or constriction of pre- and postcapillary vessels occurring simultaneously to variable and disproportionate amounts with variable, simultaneous, inter-related time-course patterns of behavior. These reactions would result in circulatory and filtration behavior which would resemble that found in the glomerulus of the kidney.

The A-V shunting vessels make it possible to have large quantities of blood immediately available in the peripheral tissues and, thereby, serve as local channels from which blood may be "tapped" by the capillaries to meet cellular requirements. Plasma skimming and axial flow of erythrocytes make it possible also for the capillaries to tap only plasma or erythrocytes in quantities to satisfy oxygen and other particular requirements of the living cells supplied by the particular capillary system.

Certain rheoplethysmographic data obtained for the finger tip of intact man indicate independent constriction of the precapillary vessels (arterioles and arteries) and the postcapillary vessels (venules and small veins) in support of the hypothesis above. The magnitude of these reactions has not been defined precisely nor have the respective vessels involved been sharply identified and quantitated. Therefore, the terms pre- and postcapillary vessels are employed in general sense. The A-V anastomoses require special considerations when the data presented below are evaluated. The pre- and postcapillary constrictor and dilator responses, although discordant in direction of change and/or magnitude of change, and although they appear to be independent, must be, and probably are, highly integrated processes.

Among the evidence for "independent" or discordant pre- and postcapillary digital vascular activity are:

1. As indicated above, noradrenalin, in the doses used, produced greater constriction of the precapillary vessels than of the postcapillary ones. There was considerable reduction in digital flow after the administration of noradrenalin with relatively little increase in tone of the collecting vessels.

2. When the collecting cuff was maintained under 40 to 60 mm. Hg pressure or more for several minutes, the digit decreased in volume, at times, by an amount as great as, or greater than, the gain in volume following inflation of the cuff. However, when the volume of the digit was at a minimum with the collecting cuff still inflated, the rate of digital inflow apparently was not reduced markedly. Therefore, the decrease in digital volume was due mainly to constriction of the postcapillary collecting vessels with relatively little constriction of the precapillary vessels.

3. The simultaneous time courses of the volume pulse waves, alpha, beta, and gamma deflections, $I_V$, $I_R$, $O_V$, and $O_R$ reflect the independent behavior of the pre- and postcapillary vessels. For example, rheoplethysmograms, at times, showed the total volume of the digit to be declining, as indicated by the descending limb of the beta or gamma deflections; at the same time, an ascending limb of an alpha deflection was inscribed with the magnitude of the pulse wave increasing and $I_V$ and $I_R$ increasing. For this type of RPG to develop, the venous or postcapillary vessels must constrict at the same time the precapillary (arterial and arteriolar) vessels must dilate. On the other hand, rheoplethysmograms were encountered, in which there was an ascending beta or gamma deflection with a simultaneous descending alpha deflection, decreasing volume pulse wave, and decreasing $I_V$ and $I_R$. For this to happen, there must be dilatation of the postcapillary collecting vessels with simultaneous precapillary vasoconstriction. It had been evident throughout many years of investigations in digital plethysmography and rheoplethysmography that the simultaneous time courses of the alpha,

beta, and gamma deflections indicated independent or discordant constriction or dilatation of the pre- and postcapillary vessels. More recent developments in rheoplethysmography, with satisfactory recording of $I_V$, $O_V$, $I_R$, and $O_R$ traces, have made possible measurements of the time courses of inflow and outflow and, in turn, the behavior of the pre- and postcapillary vessels through which digital flow occurs. The simultaneous interrelationships of these various parameters will be presented in greater detail in another report.

## DISCUSSION

These studies show the usefulness of the rheoplethysmogram in the study, not only of the circulation of the digits, but of any organ that lends itself to the type of isolation necessary for rheoplethysmographic experiments. Rheoplethysmography also makes it possible to study the pre- and postcapillary vessels, the rate of the basal component of pulsatile flow, the variations in the behavior of A-V shunts, the action of drugs, and the influence of psychogenic, neurogenic, and local physical factors, and the like upon digital blood flow. With more extensive use of, and better acquaintance with, the method, more detailed information should accumulate concerning the digital circulation.

The rheoplethysmogram showed constriction and increase in tone of the precapillary vessels produced by inflation of the collecting cuff. For example, one subject, Subject No. 4, had a mean rate of digital flow at which, if the mean rate were maintained, 78 seconds would be required for a volume of blood equal to her digital volume to flow through the digit; but by 3 pulse beats after release of the collecting cuff the mean rate had declined to a level at which 171 seconds would be required for a volume of blood equal to her digital volume to flow through the digit. These times are designated as mean times of "turnover" of digital blood. In Subject No. 1, the mean turnover time immediately after inflation of the collecting cuff was 195 seconds, but was increased to 774 seconds after prolonged inflation of the cuff. In Subject No. 5, the mean turnover time changed from 159 to 276 seconds because of inflation of the collecting cuff. That of Subject No. 7, however, changed from 120 to 101 seconds because of inflation of the collecting cuff. This latter subject experienced a slight dilatation of the precapillary vessels. However, this subject's digit decreased in volume while the collecting cuff was inflated, evidence again of discordant pre- and postcapillary vascular behavior and further evidence of increase in venous tone with venoconstriction accounting for the decrease in total volume of the digit. This subject reveals the complex nature and the variability of the reaction noted throughout these as well as many other experiments on digital flow. Most of the differences were merely quantitative, however. The response of the subjects varied in magnitude, rate, and pressure against which the digital vessels decreased in volume while the collecting cuff remained inflated. Almost all subjects showed a decrease in total digital volume to some extent, provided the pressure in the collecting cuff was not too great and the experiment was not repeated at too short an interval of time. When the studies were repeated under the same experimental conditions, each subject tended to have essentially the same RPG and same response to stimuli. Many factors, including the mental state, state of health, fatigue, and others, of which some are not always discernible, seem to influence the day-to-day findings.

That venomotor tone may increase when intravenous pressure increases is evident from simple gross inspection of the superficial vessels of the feet and legs when man stands erect from a supine position. Pressure in the veins of the feet and legs is increased, and this increase in pressure is supported in part by an increase in tone of the smooth muscle of the veins of the legs and feet. Surely the veins are not passively dilated to the maximal extent by the high intravenous pressure found when one stands. If they were, not only would the venous valves become insufficient, but man would probably faint more frequently on standing suddenly. It appears that the digital veins responded in this same fashion to inflation of the collecting cuff. Immediately after inflation of the collecting cuff the digital veins distended under increased pressure, but they began to constrict as tone increased. The degree of digital venoconstriction varied a great deal.

The four different types of curves of total digital volume described in this report emphasize again the possible inaccuracies that may develop if the slopes of the volume curve of the plethysmogram are not measured properly to obtain mean rates of digital blood flow. When the rise of the volume curve follows a straight line for several pulse cycles, it is easy to obtain an accurate mean rate of flow; but when the volume curve progressively decreases in slope within the first pulse cycle after inflation of the collecting cuff, the use of a single slope to measure the mean rate of blood flow becomes hazardous.[2] Such difficulties account for some of the discrepancies in the literature.

The alpha, beta, and gamma deflections exist constantly for the digits and probably for all organs. They apparently are related in part to pre- and postcapillary vasoconstriction with integrated function similar to that of the afferent and efferent arterioles of the glomeruli of the kidneys. These deflections probably reflect phasic changes in the rate of capillary filtration in the digits with associated variations in the quantity, at least, of the nutritional extracellular fluid bathing the cells. These intermittent variations in pre- and postcapillary constriction and dilatation coincided in time with the intermittent flow of lymph described by McMaster.[18] The importance of such variations in pre- and postcapillary vasoconstriction and dilatation in maintaining the circulation and supplying the tissues with blood and interstitial fluid in order to maintain cellular health and function remains to be clarified. Drugs which influence the state of blood vessels, such as noradrenalin, apparently influence the relative variations in pre- and postcapillary vascular activity. Unfortunately, the postcapillary vascular action of noradrenalin is often ignored when the effects of the drug are considered. The action of drugs probably varies with dosage, state of circulation, vascular disease, and duration of administration. These and many other factors need investigation.

## SUMMARY

It has been shown that partial obstruction to digital venous outflow resulted in slight increase in tone and constriction of the precapillary digital vessels

and from slight to marked increase in postcapillary vascular tone and constriction. The digital veins were among the postcapillary vessels. The mechanism for the increase in venomotor tone as well as the tone of the other vessels remains unknown.

Four types of volume-time course curves for the initial pulse cycles after inflation of the digital collecting cuff were described. These included the patterns for (1) the resting, relaxed state of the digital vessels, (2) the dilated state, (3) the constricted state (Types A and B), and (4) the obstruction to digital flow. These curves have significant rheoplethysmographic, physiologic, and pathologic connotations.

Noradrenalin was found to constrict the postcapillary digital vessels, including the veins, but the veins were not constricted as much as the precapillary vessels for the doses used.

The orienting reflex, psychogenic and neurogenic reflexes, and deep inspiration were found to produce constriction of the postcapillary vessels, including the digital veins, as well as the precapillary vessels.

It was found rheoplethysmographically that the pre- and postcapillary vessels constrict and/or dilate discordantly or disproportionately in the same manner described for the afferent and efferent arterioles of the kidney. This phenomenon probably exists for other organs as well. The phenomenon is most likely concerned with the regulation of water and solute loss into, and absorption from, the intercellular spaces, and with interstitial fluid circulation and lymph flow in healthy and diseased states.

The value of digital rheoplethysmography in the study of the peripheral circulation was further emphasized by these observations.

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# CHRONIC SODIUM CHLORIDE CHALLENGE STUDIES IN MAN

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IN RECENT years increasing emphasis has been placed by many investigators on the interrelationship of extracellular fluid volume and renal sodium excretion. In recumbent subjects, expansion of this volume leads to a sodium diuresis[1] accompanied by a fall in aldosterone excretion.[2] Crawford and Ludemann[3] have described an important qualifying feature. A time lag in natruresis follows the infusion of intravenous saline in man. Sodium excretion, in spite of initial extracellular fluid expansion, increases only moderately after 3½ hours. Only with prolonged infusions of 2.5 per cent saline can pronounced sodium excretion occur.[4]

Leaf and Couter[5] have described a similar lag in the renal excretion of sodium in patients who ingest high constant sodium loads; sodium urinary output lags behind intake for several days, even though there is a marked increase in extracellular volume. Their studies revealed also a time lag in the renal conservation of sodium when sodium intake was reduced. Some years ago, Gamble[6] depicted the same lag phenomenon in infants, and described an extensive oscillation of extracellular fluid and of electrolyte balance in an ebb and flow which extended over periods of many days.

Further light has been shed on this time lag phenomenon of sodium excretion by Ladd,[7] who found that prehydration with water 8 to 13 hours before intravenous saline infusions eliminated the delay and effected a copious natruresis. Recently, Smith[8] has postulated that the inhibitory effect of prehydration on the sodium retaining system remains subliminal but undergoes progressive potentiation with time so that a saline infusion induces prompt natruresis.

The cyclical fluctuations in sodium balance observed by Gamble, and the observations of Leaf and Couter may well prove ascribable to just such a mechanism in which there results not only potentiation of subliminal stimuli inhibiting sodium retention, but, also, a similar potentiation exciting it. These, alternately occurring, may reflect the gradual attainment of equilibrium. If the time factor is as important as it appears to be in the potentiation of inhibitory and excitatory stimuli governing sodium retention, increasing the daily sodium intake from a lower to a higher level should exaggerate the positivity

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and negativity of the resultant sodium balance fluctuations prior to equilibration at the higher intake. The studies to be reported here support this concept.

## MATERIAL AND METHODS

Thirteen patients were studied. All except one (L. P.) were free from any apparent endocrine, cardiovascular or renal disease. Of these, 12 were "orthopedic" or "plastic"—underwent surgical procedures which necessitated plaster casting. One patient (E. H.) suffered from a spinal artery thrombosis, and the thirteenth (L. P.) suffered from biliary cirrhosis and hemochromatosis. *All patients were at bed rest and relatively motionless.* No patient was studied until a minimum of at least 12 days had elapsed after surgical or casting procedure, with the exception of N. C., who underwent a minor surgical procedure under local anesthesia during the course of the higher sodium intake studies. The patients ranged widely as to age and weight. All were studied during cool weather or in air-conditioned rooms and in no instance did the temperature in the rooms vary more than 8 degrees throughout each study period. All patients were normotensive; none of the females were studied during, or just prior to, menses; and all were afebrile. Laboratory data were obtained to confirm the absence of anemia, renal disease, or electrolyte distortion. Clinical data including those of the patient with biliary cirrhosis are given subsequently.

Each individual was given a calculated sodium intake of 70 mEq. per square meter surface area for 2 to 9 days. This was provided by supplementing sodium restricted diets (average sodium content: 11 mEq.; average potassium content: 67 mEq.) with an additional calculated amount of sodium chloride distributed equally in gelatin capsules and taken after each of the 3 meals. This was then increased to a daily sodium intake of 120 mEq. of sodium per square meter surface area in all cases except F. McG., whose intake was increased to 170 mEq., and L. P., whose intake was increased to 130 mEq. Daily sodium intakes ranged from 87 to 141 mEq. prior to loading, and from 149 mEq. to 299 mEq. after loading. Higher daily intakes could not be tolerated per os. Four selected low sodium diets, each containing 1,950 calories, 260 Gm. carbohydrate, 65 Gm. protein, and 80 Gm. fat, were alternated throughout the entire study, and the diets were intermittently ashed and analyzed to ensure constancy of sodium content. In all instances the patients were cooperative and nearly always consumed the entire meal. Water was allowed ad libitum and intakes were measured.

Urine was collected for 24 hour periods (calculated from exactly 8:00 A.M. to 8:00 A.M.) and volume measurements were obtained. Sodium and potassium contents were measured using a Coleman Flame Photometer.

Stools were pooled for 3 to 4 day periods, with the exception of the first day on the higher sodium intake, when single 24 hour collections were made. All stools were dried to constant weight and pulverized. Aliquots were ashed and sodium content determined by flame photometry. Fecal sodium excretion was determined in all except 4 patients.

Inasmuch as it was not possible to measure the daily sodium excretion in perspiration, *true* balances could not be determined. Many of these patients were in large body casts and they differed widely in age and body surface area. Because these unmeasurable and perhaps variable sodium losses may have been in some instances significant, the mean values for sodium excretion during the period of observation on any single sodium load were taken as a base line from which fluctuations of excretion could be measured. The mean value for the preloading period and the postloading period were calculated independently. The mean daily excretion permits comparison of fluctuations in sodium excretion between individuals and in any single individual on different intakes. "Balances" calculated from urine and fecal excretion data alone as related to intake, excluding sodium loss from sweat and other secretions, are identical in shape and configuration with the calculated curves (Fig. 1).

It may well be that curves of the fluctuation of sodium excretion from mean excretion represent a closer approximation of *true* sodium balance than those obtained from the measured difference between sodium load and sodium fecal and urine excretion alone, since the patients' weights were constant and since variable losses of sodium from sweat and other

secretions may be in this way to some degree corrected. Gamble[6] has made such an assumption in his studies on infants. In full awareness that this is not a measured balance, fluctuations of excretion from the base line have been referred to throughout as "changes in sodium balance." This technique for approximating *true* balances does in no way detract from the validity of the observations to be reported of the relationship of load to fluctuation of excretion.

*Clinical Data.—*

1. F. W. was a 14-year-old schoolboy admitted for tendon transplants and arthrodesis of the ankle to correct deformities secondary to poliomyelitis. His weight was 82 pounds and his height 4 feet 9 inches with a surface area of 1.24 sq. M.

2. B. M. was an 11-year-old schoolgirl admitted for surgical arrest of femoral and tibial epiphyses in one leg. Her weight was 98 pounds and her height 4 feet 7 inches with a calculated surface area of 1.29 sq. M.

3. S. V. was an 11-year-old schoolgirl admitted for orthopedic correction of a foot drop secondary to myelosclerosis. Treatment consisted of casting and spinal fusion. Her weight was 100 pounds and her height 5 feet 1 inch with a surface area of 1.41 sq. M.

4. J. P. was a 37-year-old housewife with Pott's Disease necessitating body casting. Her weight was 117 pounds and her height 5 feet 3 inches with a surface area of 1.54 sq. M.

5. E. H. was a 50-year-old, 5 feet 10 inches housewife weighing 130 pounds whose surface area was 1.56 sq. M. She was hospitalized for treatment after incurring a spinal artery thrombosis at the twelfth thoracic level with momentary bladder paresis and minimal weakness in one leg. An indwelling catheter was in place throughout the period of study.

6. W. L. was a 30-year-old white man admitted for reduction of several fractures of the femur. The balance studies were not begun until 5 months after his last operation. His weight was 113 pounds and his height 5 feet 8 inches with a surface area of 1.6 sq. M.

7. J. D. was a 16-year-old schoolgirl admitted for triple arthrodesis of the left ankle. Her weight was 137 pounds and her height 5 feet 6 inches with a surface area of 1.72 sq. M.

8. F. S. was a 60-year-old laborer admitted for treatment of skull and pelvic fractures. The balance studies were not begun until 2 months after admission, when his general condition was well stabilized. His weight was 160 pounds and his height 5 feet 7 inches with a surface area of 1.84 sq. M.

9. K. R. was a 21-year-old x-ray technician who was admitted for a triple arthrodesis of his ankle to correct a congenitally spastic foot. His weight was 170 pounds and his height 6 feet with a surface area of 2.0 sq. M.

10. D. M. was a 53-year-old postmenopausal office worker admitted for treatment of a fractured ankle. A mild hypertension of 150/80 mm. Hg was noted on admission, which on bed rest alone fell to 130/80 mm. Hg. Her weight was 220 pounds and her height 5 feet 3½ inches with a surface area of 2.02 sq. M.

11. N. C. was a 43-year-old printer who was hospitalized for skin grafting to an old scar secondary to a leg fracture. His height was 5 feet 11 inches and his weight 200 pounds with a surface area of 2.09 sq. M.

12. F. McG. was a 59-year-old laborer admitted for treatment of multiple fractures of the pelvis. The balance studies were not begun until 12 days after surgery had elapsed. His weight was 150 pounds and his height 5 feet 6 inches with a surface area of 1.76 sq. M.

13. L. P. was a 45-year-old man admitted for treatment of biliary cirrhosis and hemochromatosis with moderate peripheral edema, but no demonstrable ascites. The diagnosis was verified by punch liver biopsy. Esophageal varices were demonstrated by x-ray. Blood cholesterol was 570 mg. per cent, total bilirubin 6.2 mg. per cent with a direct bilirubin of 2.6 mg. per cent; cephalin flocculation was 3 plus at 24 hours and 4 plus at 48 hours. Alkaline phosphatase was 35 King-Armstrong units per 100 ml. Concentration, dilution and PSP studies of renal function were normal. Total protein was 6.0 with 1.8 Gm. albumin. His initial weight was 178 pounds; his final weight was 176 pounds.

In all patients studied, the hemogram, plasma sodium, potassium, chloride, and NPN concentration, as well as urinalyses, were normal before and after studies.

## RESULTS

*Sodium Excretion.*—A most unexpected observation was the marked variation in sodium excretion demonstrated by many patients in spite of the constant intake. Mean sodium excretion increased after sodium intakes were raised, as did variation of excretion. (Table I.) In the patients of larger surface area who received the larger intakes, these variations were at times extreme. F. S. revealed a change of excretion in one instance of 124 mEq. per 24 hours. L. P. demonstrated changes in daily excretion ranging as high as 220 mEq. Fecal excretion of sodium on the other hand was practically negligible; it reached a maximum of 8 mEq. per 24 hours in only one instance. Daily sodium over-all gain or loss data (calculated in relation to mean excretion) are provided in Fig. 1. The patients are ranked from top to bottom in the order of magnitude of their surface area and sodium intake. The patient with cirrhosis (L. P.) has been added at the bottom, and was included in this report to illustrate the wide range of sodium excretion possible in hospitalized patients. The data from this patient have been *excluded* from all the calculations involved in the observations reported from the others. The absolute variation in daily sodium gain or loss is smaller in patients of smaller surface area presented at the top of Fig. 1. Smaller patients received proportionately smaller sodium intakes. In the sodium loading studies on the larger patients, a definite pattern emerges of alternating fluctuations of balance, roughly sinusoidal in shape, and irregular in interval.

The standard deviation of total daily sodium excretion was determined in all patients for both preloading and postloading studies. These data provide numerical indices of the range of variation of excretion. In each patient (Fig. 1) this value increased after the sodium intake was elevated. For all patients the mean standard deviation on the lower intake was 14.6 mEq. of sodium per 24 hours; the mean for the higher intake was 39.1 mEq.

The amplitude of the oscillations of daily sodium excretion was a linear function of intake. When the standard deviations of both pre- and postload sodium excretion were plotted against load for all normal patients (the patient with cirrhosis was excluded), this correlation was clear (Fig. 2). The correlation coefficient was 0.82 with a "t" value of 6.72 and a "p" value of less than 0.001. When the preload data which comprised less than 4 days of study were omitted, the correlation coefficient of the relationship of the standard deviation of sodium excretion and sodium intake was *still* high, being 0.73, with a "t" value of 3.99, and a "p" value of less than 0.01. The variance of excretion was predictable in all normal patients, independent of their size, and was a linear function of load.

*Water Excretion.*—In view of the recent proposals of Berliner and others,[9, 10] who direct attention to the predominant role of sodium upon urine concentration mechanisms in the distal nephron, an attempt was made to correlate urine flow with the varying sodium excretion. In view of the allowance of water ingestion ad libitum in this series, it might be anticipated that water excretion would bear little relationship to this ion. Although in the majority of patients the correlation was poor, in 4 it was extremely high. (Table II and

TABLE I.

| FLUID INTAKE (ML./24 HRS.) | URINE VOLUME (ML./24 HRS.) | URINE Na (mEq./24 HRS.) | FECAL Na (mEq./24 HRS.) | URINE K (mEq./24 HRS.) | Na:K RATIO | VARIATION Na EXCRETION FROM MEAN EXCRETION (mEq./24 HRS.) |
|---|---|---|---|---|---|---|
| *F. W.*— | | | | | | |
| Daily Na intake, 86.8 mEq.; mean daily Na excretion, 59.1 mEq. | | | | | | |
| — | 350 | 62 | — | 31 | 0.4 | − 3 |
| | 560 | 56 | | 36 | 1.5 | + 3 |
| Daily Na intake, 148.8 mEq.; mean daily Na excretion, 143.0 mEq. | | | | | | |
| — | 600 | 121 | — | 27 | 4.5 | + 22 |
| | 540 | 116 | | 16 | 7.2 | + 27 |
| | 920 | 243 | | 39 | 6.3 | −100 |
| | 530 | 122 | | 28 | 4.4 | + 21 |
| | 500 | 112 | | 26 | 4.4 | + 31 |
| *B. M.*— | | | | | | |
| Daily Na intake, 91 mEq.; mean daily Na excretion, 67.8 mEq. | | | | | | |
| 1175 | 1210 | 60 | — | 50 | 1.2 | + 7 |
| 1865 | 800 | 73 | | 31 | 2.4 | − 7 |
| Daily Na intake, 156 mEq.; mean daily Na excretion, 119.6 mEq. | | | | | | |
| 1300 | 680 | 119 | — | 75 | 1.6 | + 0 |
| 1850 | 670 | 128 | | 51 | 2.5 | − 9 |
| 1000 | 700 | 120 | | 65 | 1.8 | − 1 |
| 1400 | 530 | 99 | | 63 | 1.6 | + 20 |
| 1900 | 1050 | 130 | | 45 | 2.9 | − 10 |
| *S. V.*— | | | | | | |
| Daily Na intake, 98.7 mEq.; mean daily Na excretion, 82.2 mEq. | | | | | | |
| — | 670 | 69 | 0.2 | 43 | 1.0 | + 13 |
| | 520 | 86 | 0.2 | 23 | 2.0 | − 4 |
| | 970 | 91 | 0.2 | 38 | 2.4 | − 9 |
| Daily Na intake, 169.2 mEq.; mean daily Na excretion, 159.5 mEq. | | | | | | |
| — | 1040 | 152 | 0.1 | 56 | 2.7 | + 7 |
| | 640 | 155 | 0.1 | 29 | 5.3 | + 4 |
| | 500 | 143 | 0.1 | 28 | 5.1 | + 16 |
| | 550 | 123 | 0.1 | 26 | 4.7 | + 36 |
| | 680 | 193 | 0.1 | 31 | 6.2 | − 34 |
| | 730 | 206 | — | 28 | 7.4 | − 46 |
| | 490 | 144 | — | 24 | 6.0 | + 15 |
| *J. P.*— | | | | | | |
| Daily Na intake, 107.8 mEq.; mean daily Na excretion, 113.6 mEq. | | | | | | |
| 1300 | 1505 | 119 | 2.3 | 32 | 6.0 | − 8 |
| 2000 | 2115 | 99 | 2.3 | 27 | 3.6 | + 12 |
| 1325 | 2020 | 115 | 2.3 | 28 | 5.5 | − 4 |
| — | 1940 | 122 | 2.6 | 11 | 10.9 | − 11 |
| 1750 | 1717 | 123 | 2.6 | 14 | 8.8 | − 12 |
| 1800 | 1580 | 121 | 2.6 | 14 | 8.7 | − 10 |
| 1700 | 1688 | 99 | 4.6 | 13 | 7.4 | + 10 |
| 1620 | 1138 | 87 | 4.6 | 8 | 9.2 | + 21 |
| Daily Na intake, 184.8 mEq.; mean daily Na excretion, 160.3 mEq. | | | | | | |
| 2000 | 1994 | 127 | 4.6 | 13 | 9.7 | + 28 |
| 1600 | 1568 | 143 | 0.3 | 23 | 6.1 | + 17 |
| 1800 | 1910 | 165 | 0.3 | 13 | 12.3 | − 5 |
| 1400 | 1252 | 166 | 0.3 | 17 | 9.9 | − 6 |
| 1800 | 1555 | 148 | 0.0 | 22 | 6.8 | + 12 |
| 1500 | 1742 | 207 | 0.0 | 26 | 7.9 | − 47 |
| *E. H.*— | | | | | | |
| Daily Na intake, 109.2 mEq.; mean daily Na excretion, 125.5 mEq. | | | | | | |
| — | 5490 | 148 | 0.2 | 74 | 2.0 | − 23 |
| | 1930 | 96 | 0.2 | 37 | 2.6 | + 29 |
| | 1920 | 132 | 0.2 | 42 | 3.1 | − 7 |
| Daily Na intake, 187.2 mEq.; mean daily Na excretion, 184.5 mEq. | | | | | | |
| — | 1740 | 194 | 0.3 | 57 | 3.4 | − 10 |
| | 1960 | 192 | 0.8 | 51 | 3.8 | − 8 |

TABLE I (CONT'D.)

| FLUID INTAKE (ML./24 HRS.) | URINE VOLUME (ML./24 HRS.) | URINE Na (mEq./24 HRS.) | FECAL Na (mEq./24 HRS.) | URINE K (mEq./24 HRS.) | Na:K RATIO | VARIATION Na EXCRETION FROM MEAN EXCRETION (mEq./24 HRS.) |
|---|---|---|---|---|---|---|
| | 2240 | 172 | 0.8 | 55 | 3.1 | + 12 |
| | 1840 | 139 | 0.8 | 48 | 2.9 | + 45 |
| | 3520 | 176 | 0.8 | 49 | 3.6 | + 8 |
| | 1625 | 176 | 0.8 | 41 | 4.3 | + 8 |
| | 2845 | 265 | 0.8 | 54 | 4.9 | − 81 |
| | 1460 | 156 | 0.8 | 50 | 3.1 | + 28 |
| *W. L.*— | | | | | | |
| Daily Na intake, 112 mEq.; mean daily Na excretion, 131.4 mEq. | | | | | | |
| — | 1260 | 130 | — | 65 | 2.0 | + 2 |
| | 1156 | 133 | | 53 | 2.5 | − 2 |
| Daily Na intake, 192 mEq.; mean daily Na excretion, 192.1 mEq. | | | | | | |
| — | 1521 | 175 | — | 54 | 3.2 | + 17 |
| | 1760 | 195 | | 62 | 3.1 | − 3 |
| | 2170 | 181 | | 54 | 3.3 | + 11 |
| | 1480 | 228 | | 59 | 3.8 | − 36 |
| | 1470 | 181 | | 56 | 3.2 | + 11 |
| *J. D.*— | | | | | | |
| Daily Na intake, 122.2 mEq.; mean daily Na excretion, 129.2 mEq. | | | | | | |
| — | 1145 | 137 | — | 49 | 2.8 | − 8 |
| | 1680 | 121 | | 45 | 2.3 | + 8 |
| Daily Na intake 207.7 mEq.; mean daily Na excretion, 177.03 mEq. | | | | | | |
| — | 1120 | 124 | — | 47 | 2.1 | + 53 |
| | 1280 | 155 | | 28 | 5.5 | + 22 |
| | 1300 | 224 | | 52 | 4.3 | − 47 |
| | 1220 | 217 | | 50 | 4.3 | − 40 |
| | 1220 | 184 | | 37 | 5.0 | − 7 |
| | 880 | 132 | | 31 | 4.2 | + 45 |
| | 1080 | 203 | | 46 | 4.5 | − 26 |
| *F. S.*— | | | | | | |
| Daily Na intake, 128.8 mEq.; mean daily Na excretion, 134.5 mEq. | | | | | | |
| — | 3020 | 142 | 0.5 | 41 | 3.5 | − 8 |
| | 3480 | 125 | 0.5 | 42 | 3.0 | + 9 |
| | 2360 | 135 | 0.5 | 34 | 3.9 | − 1 |
| Daily Na intake, 220.8 mEq.; mean daily Na excretion, 231.4 mEq. | | | | | | |
| — | 2430 | 182 | 0.2 | 32 | 5.8 | + 49 |
| | 2900 | 305 | 1.4 | 42 | 7.3 | − 75 |
| | 2710 | 230 | 1.4 | 42 | 5.5 | 0 |
| | 3040 | 242 | 1.4 | 36 | 6.6 | − 12 |
| | 1920 | 209 | 0.4 | 38 | 5.4 | + 22 |
| | 2210 | 223 | 0.4 | 46 | 4.8 | + 8 |
| | 1450 | 223 | 0.4 | 48 | 4.7 | + 8 |
| *E. R.*— | | | | | | |
| Daily Na intake, 140 mEq.; mean daily Na excretion, 131.8 mEq. | | | | | | |
| — | 1430 | 130 | 0.8 | 85 | 1.5 | + 1 |
| | 1320 | 120 | 0.8 | 68 | 1.8 | + 11 |
| | 1535 | 143 | 0.8 | 38 | 3.7 | − 12 |
| Daily Na intake, 240 mEq.; mean daily Na excretion, 246.1 mEq. | | | | | | |
| — | 1138 | 196 | 3.1 | 64 | 3.0 | + 49 |
| | 2282 | 309 | 0.6 | 71 | 4.4 | − 63 |
| 1420 | 2292 | 272 | 0.6 | 80 | 3.4 | − 26 |
| | 1610 | 206 | 0.6 | 86 | 2.4 | + 39 |
| | 1917 | 252 | 1.1 | 79 | 3.2 | − 7 |
| | 1912 | 274 | 1.1 | 94 | 2.9 | − 28 |
| 1900 | 1763 | 268 | 1.1 | 88 | 3.0 | − 23 |
| | 1830 | 255 | 2.8 | 71 | 3.6 | − 12 |
| | 890 | 172 | 2.8 | 68 | 2.5 | + 72 |
| *J. M.*— | | | | | | |
| Daily Na intake, 141.4 mEq.; mean daily Na excretion, 144.9 mEq. | | | | | | |
| 3135 | 1852 | 81 | 3.1 | — | — | + 61 |
| 2700 | 2085 | 118 | 3.1 | | | + 24 |

TABLE I (CONT'D.)

| FLUID INTAKE (ML./24 HRS.) | URINE VOLUME (ML./24 HRS.) | URINE Na (mEq./24 HRS.) | FECAL Na (mEq./24 HRS.) | URINE K (mEq./24 HRS.) | Na:K RATIO | VARIATION Na EXCRETION FROM MEAN EXCRETION (mEq./24 HRS.) |
|---|---|---|---|---|---|---|
| 2900 | 2072 | 122 | 3.1 | | | + 20 |
| 2885 | 2496 | 175 | 3.1 | | | − 33 |
| 3060 | 3593 | 139 | 2.5 | | | + 3 |
| 3060 | 2407 | 127 | 2.5 | | | + 15 |
| 3300 | 2914 | 183 | 2.5 | | | − 40 |
| 3110 | 2716 | 193 | 2.5 | | | − 51 |
| 3160 | 2592 | 141 | 3.2 | | | + 0 |
| Daily Na intake, 242.4 mEq.; mean daily Na excretion, 233.4 mEq. | | | | | | |
| 3230 | 2375 | 160 | 0.3 | — | — | + 73 |
| 3380 | 2517 | 211 | 0.3 | | | + 22 |
| 3380 | 3036 | 270 | 0.3 | | | − 37 |
| 3600 | 1930 | 181 | 0.3 | | | + 52 |
| 3180 | 3057 | 287 | 0.3 | | | − 54 |
| 2980 | 2926 | 263 | 8.1 | | | − 38 |
| 3380 | 2844 | 244 | 8.1 | | | − 19 |
| *N. C.*— | | | | | | |
| Daily Na intake, 146.3 mEq.; mean daily Na excretion, 154.4 mEq. | | | | | | |
| 4475 | 2830 | 174 | 5.8 | 61 | 2.9 | − 25 |
| 5025 | 3044 | 141 | 5.8 | 45 | 3.1 | + 8 |
| 4600 | 2790 | 150 | 5.8 | 45 | 3.4 | − 1 |
| 4650 | 3347 | 140 | 5.3 | 42 | 3.4 | + 9 |
| 5150 | 3992 | 160 | 5.3 | 37 | 4.3 | − 11 |
| 4200 | 2942 | 127 | 5.3 | 37 | 3.4 | + 22 |
| Daily Na intake, 250.8 mEq.; mean daily Na excretion, 246.8 mEq. | | | | | | |
| 4400 | 2775 | 213 | 5.3 | 42 | 5.1 | + 29 |
| 4500 | 2946 | 258 | 4.3 | 47 | 5.5 | − 16 |
| 5000 | 3837 | 299 | 4.3 | 50 | 6.0 | − 57 |
| 4900 | 3060 | 156 | 0.0 | 42 | 3.7 | + 91 |
| 4900 | 3278 | 269 | 0.0 | 45 | 5.9 | − 22 |
| 3400 | 2250 | 248 | 0.0 | 39 | 6.3 | − 2 |
| 3600 | 2072 | 278 | 0.0 | 42 | 6.6 | − 31 |
| 4175 | 2640 | 239 | 0.0 | 42 | 5.7 | + 8 |
| *FMcG.*— | | | | | | |
| Daily Na intake, 123.2. mEq.; mean daily Na excretion, 124.8 mEq. | | | | | | |
| 3200 | 2304 | 74 | 1.9 | 47 | 1.6 | + 49 |
| 3500 | 3144 | 118 | 1.9 | 50 | 2.4 | + 5 |
| 5300 | 2437 | 155 | 1.9 | 35 | 4.5 | − 32 |
| 2600 | 2365 | 136 | 0.5 | 47 | 2.9 | − 12 |
| 3500 | 2397 | 117 | 0.5 | 35 | 3.4 | + 7 |
| 3950 | 2017 | 121 | 0.5 | 31 | 3.9 | + 3 |
| 4050 | 1968 | 147 | 0.5 | 28 | 5.2 | − 22 |
| 2500 | 2270 | 121 | 0.9 | 29 | 4.2 | + 3 |
| 1650 | 1775 | 124 | 0.9 | 37 | 3.3 | − 0 |
| Daily Na intake, 299.2 mEq.; mean daily Na excretion, 255.4 mEq. | | | | | | |
| 2350 | 2395 | 215 | 0.7 | 30 | 7.1 | + 39 |
| 3000 | 2770 | 332 | 0.4 | 31 | 7.7 | − 77 |
| 2500 | 1355 | 217 | 0.4 | 19 | 11.6 | + 38 |
| *L. P.*— | | | | | | |
| Daily Na intake, 141.4 mEq.; mean daily Na excretion, 123.7 mEq. | | | | | | |
| 4100 | 3840 | 110 | 0.9 | 28 | 1.0 | + 13 |
| 4320 | 2475 | 136 | 0.9 | 15 | 3.8 | − 13 |
| Daily Na intake, 264.0 mEq.; mean daily Na excretion, 258.1 mEq. | | | | | | |
| 4175 | 4910 | 218 | 0.9 | 12 | 3.7 | + 39 |
| 3160 | 2430 | 245 | 0.3 | 20 | 5.2 | + 12 |
| 2945 | 2915 | 349 | 0.6 | 21 | 5.7 | − 92 |
| 3210 | 1850 | 129 | 0.6 | 27 | 2.5 | +128 |
| 3000 | 3275 | 314 | 0.6 | 21 | 4.7 | − 5 |
| 3215 | 3500 | 379 | 2.5 | 16 | 6.8 | −124 |
| 3215 | 3060 | 294 | 2.5 | 18 | 5.3 | − 38 |
| 2865 | 1760 | 124 | 2.5 | 30 | 2.4 | +131 |

Fig. 3.) In no instance was mean daily water excretion significantly higher during the higher sodium intake studies than during the lower intake studies.

*Potassium Excretion.*—Potassium excretion was remarkably constant throughout. (Table I.) Because potassium intakes could not be accurately measured, potassium balances could not be determined. Gamble[6] described an increase in potassium excretion with sodium chloride loading; Leaf and Couter[5] described a decrease. In all likelihood, the small magnitude of the variation in excretion of urinary potassium here is partly accountable to the moderate content of the diet. It was totally unexpected that urinary potassium excretion could remain relatively constant in the presence of marked variation of sodium excretion.

*Sodium to Potassium Excretion Ratios.*—Numerous investigators have called attention to the Na to K ratio as an index of aldosterone excretion.[11-15] In these studies this ratio holds a high correlation with sodium excretion. Arithmetically this relationship merely reflects the constancy of potassium excretion. (Table I.) That this has physiologic significance here is speculative.

## DISCUSSION

The pattern of maintenance of constant body sodium stores in the face of chronic sodium chloride loading is the predominant observation reported in these studies. Leaf and Couter[5] and Gamble[6] in similar chronic loading studies have calculated from the chloride space that the bulk of the additional sodium chloride is retained as a virtually isotonic fluid in an expanded extracellular fluid volume. Leaf and Couter,[5] in a 3 day loading period study, expressed the belief that this expansion of extracellular fluid volume would remain fixed. Gamble[6] denied this, describing a fluctuation of extracellular fluid volume, which expanded and contracted with the continuance of the daily sodium load for longer periods. It appears likely in the studies reported here that the extracellular fluid alternately expands and contracts, accompanying the fluctuations of body sodium stores in view of the free allowance of water ingestion and the constancy of serum sodium concentration. Such an alternating pattern of extracellular fluid volume is consonant with the observations of many investigators,[16-18] including Zierler and Lilienthal[16] who described a "break-away"

TABLE II. CORRELATION COEFFICIENTS: RELATIONSHIP OF POSTLOADING WATER EXCRETION TO SODIUM EXCRETION

| PATIENT | CORRELATION COEFFICIENT |
|---|---|
| F. W. | 0.97 |
| B. M. | 0.48 |
| S. V. | 0.31 |
| J. P. | −0.14 |
| E. H. | 0.41 |
| W. L. | −0.28 |
| J. D. | 0.53 |
| F. S. | 0.47 |
| K. R. | 0.93 |
| D. M. | 0.89 |
| N. C. | 0.13 |
| L. P. | 0.95 |

excretion of sodium after initial retention in the face of continued DOCA therapy in man. Moreover, a similar initial weight gain and sodium retention and subsequent sodium excretion and weight loss phenomenon has recently been

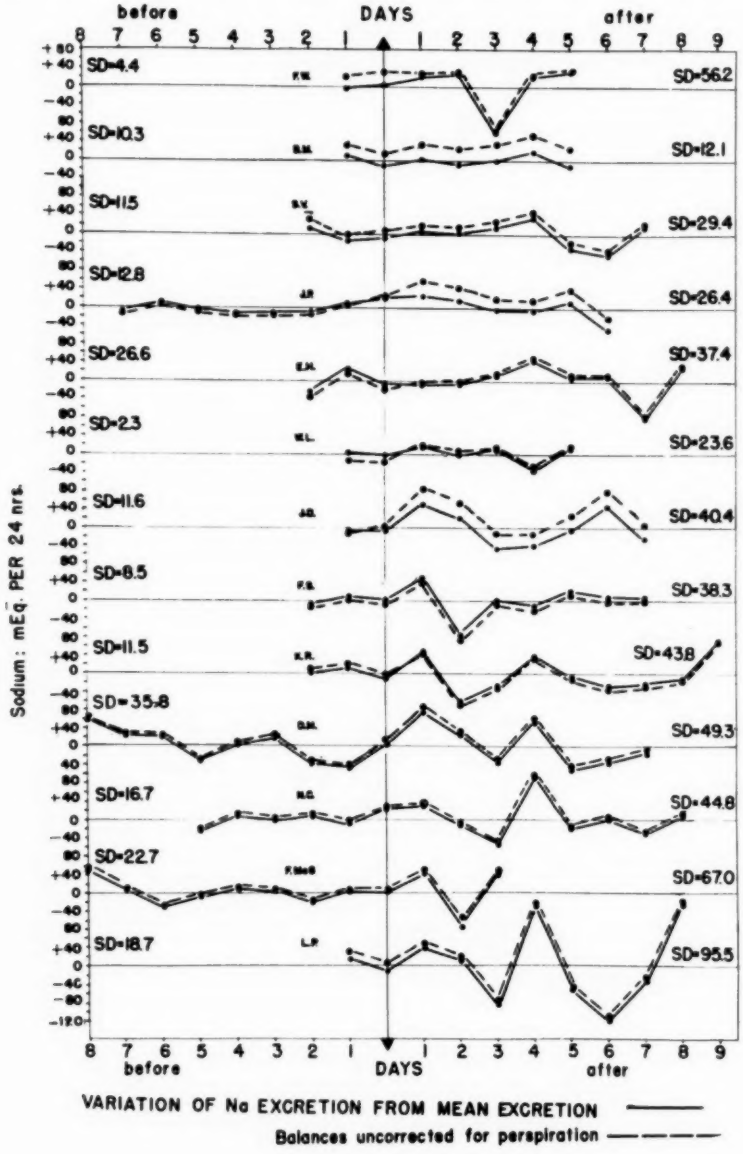


Fig. 1.

described by August and co-workers[19] in subjects receiving aldosterone. Whatever the mechanisms involved in this alternation of extracellular volume,[2] the lag phenomenon of sodium retention and excretion appears frequently in many studies of this kind.

Smith[8] has postulated that the antinatruretic system has central nervous system representation, which provides a locus for the algebraic summation of excitatory and inhibitory stimuli. Hydration of subjects 24 hours prior to a challenging infusion of saline solution evokes a marked natruretic response

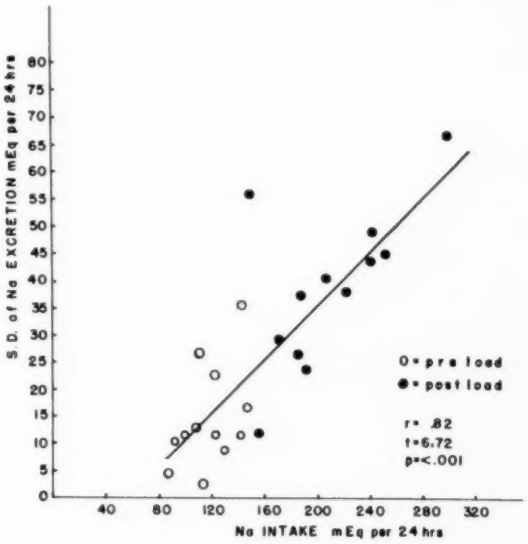


Fig. 2.

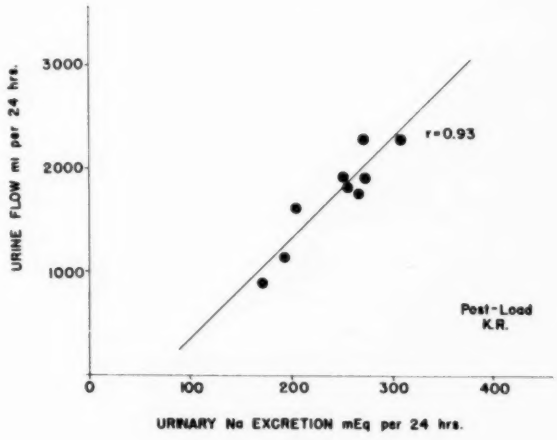


Fig. 3.

which eliminates the sodium excretion lag, a phenomenon which is not observed in nonprehydrated subjects.[7] He suggests that these responses reflect a subliminal progressively increasing "inhibition" of the sodium retaining system.

Fig. 4 provides a hypothetical representation of potentiation concepts as applied to fixed daily sodium loading studies. Utilizing any arbitrary daily sodium intake such as the given one of 3 units, the response of the daily swings

of sodium balance in the absence of potentiation and in the presence of a fixed rate of excretion can provoke only one general pattern comprising initial retention and gradual equilibration to a zero balance. In this pattern, sodium excretion gradually equals sodium intake without acceleration of the rate of excretion of the daily sodium load. By contrast, if the sodium retention mechanism is progressively inhibited with time and the rate of sodium excretion is accelerated, as Smith[8] suggests, a second pattern of fluctuant balance emerges. (Fig. 4.) This comprises an initial positivity of balance, a subsequent negativity, and, thereafter, equilibration to a persistent zero balance. In this pattern the rate of sodium excretion accelerates with time, providing the increase

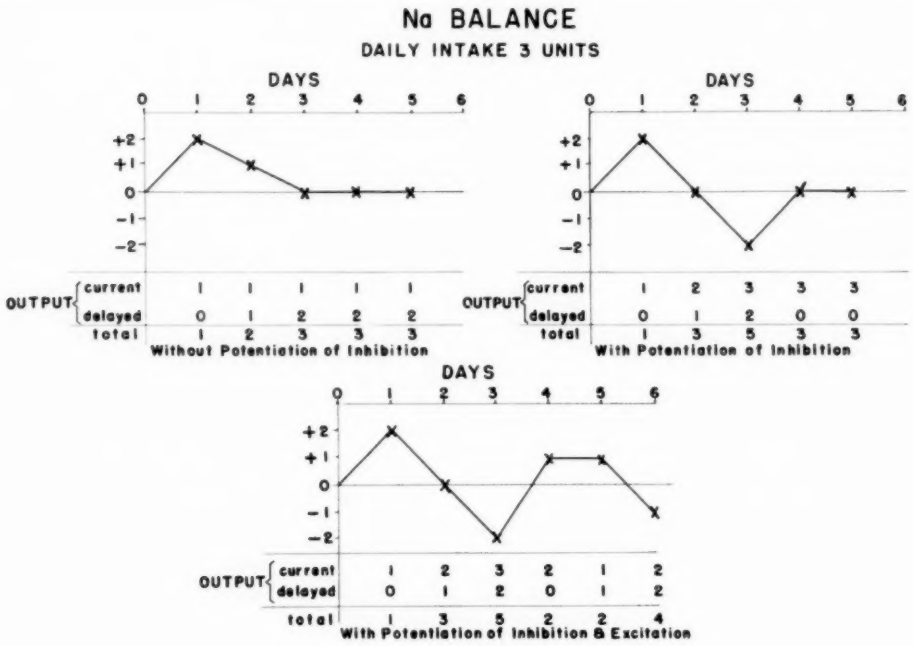


Fig. 4.

of excretion over intake which comprises the negative balance overshoot. Here a second positive sodium balance cannot occur with potentiation of inhibition alone of the sodium retaining mechanism. A third hypothesis which includes potentiation of both inhibition and excitation is necessary to provide alternating positive and negative balances as noted in our studies. Such an interpretation is suggested as a possible explanation of these cylic phases in sodium balance. This is comparable to the feed-back mechanisms common to known biologic interrelationships of one metabolic function with another. In this instance the lag of sodium excretion and the lag of sodium retention occur alternately as the acceleration of each is triggered. With the passage of time the amplitude of sodium balance swings may progressively diminish. This appears to be the case with subjects F. S., D. M., and N. C. (Fig. 1). Many of the other subjects, however, particularly K. R. and L. P., reveal no evidence

of equilibration to a zero balance after more than a week on the higher sodium intake. Studies of longer duration can shed further light upon this problem. The suggestion, nonetheless, exists that these sinusoidal alternations reflect a gradual "hunting" process, seeking zero balance with the passage of time. Furthermore, at least in the short run, it appears clear that the amplitude of the balance fluctuations is determined by the magnitude of the daily sodium load. It would also appear clear that caution must be exercised in the clinical and physiologic interpretation of 24 hour urinary sodium excretion data from subjects at bed rest. The over-all balance pattern may vary widely from one day to another.

## SUMMARY

1. In spite of a fixed sodium chloride intake, daily sodium excretion may vary widely.

2. The sodium balance pattern of larger subjects given proportionately larger sodium loads presents a sinusoidal configuration comprising an alternation of positivity and negativity in spite of a fixed sodium intake.

3. The amplitude of the oscillations of sodium excretion is a linear function of the magnitude of the daily sodium chloride load in the short run in all normal patients.

4. The gastrointestinal absorption of sodium chloride in the amounts given is practically complete; the fecal excretion of sodium is insignificant.

5. The urinary potassium excretion in spite of sodium chloride loading as described is generally constant throughout.

6. Hypothetical interrelationships of the above with current concepts of electrolyte metabolism are discussed.

7. Clinical and physiologic interpretations of single 24 hour urinary sodium excretion data must be made with caution.

We wish gratefully to acknowledge the technical assistance of Sylvia Pandolfi and Elaine Horn, the assistance of the Dietary Department of the Presbyterian–St. Luke's Hospital, specifically Betty Okada and Jane Mikkelsen, and the encouragement and criticism of Drs. Theodore B. Schwartz and John W. Clark.

## ADDENDUM*

*A Mathematical Model for Sodium Excretion.*—If we suppose that sodium excretion is controlled by a hormone, and if this hormone is itself indirectly controlled by the salt content of the extracellular fluid, we can account for the temporal behavior of the excretion of salt when the intake level is suddenly changed. To simplify matters, consider changes small enough that the system can be treated as approximately linear. It has been suggested (Strauss, p. 237[20]) that aldosterone, which inhibits sodium excretion, is itself reduced in amount as a result of increased extracellular volume. If we suppose that the extracellular volume is approximately proportional to the sodium content when relatively long periods of time are considered, it is as if the hormone content (A) were controlled by the sodium content (S). For the linear situation we would then have the following approximate equation:

$$\frac{dA}{dt} = (P - a\Delta S) - mA; \qquad (1)$$

*The addendum was contributed by Dr. Herbert D. Landahl of the Committee on Mathematical Biology of the University of Chicago, Chicago, Ill.

that is, the content of the hormone is increased by the production term $P - \alpha S$, $\alpha$ being a coefficient which measures the inhibitory effect of the extracellular volume change reflected in the change, $\Delta S$, in S from some normal level, and the hormone is eliminated metabolically or by excretion at a rate $mA$, m being a coefficient for this rate. The salt content is determined by the balance between the intake ($I_s$) and the excretion, taken here to be linear in the change, $\Delta A$, in A. If $\Delta I_s$ is the change in intake from some normal level previously maintained and is used as a reference level, the salt content is determined from

$$\frac{d\Delta S}{dt} = \Delta I_s + \beta \Delta A, \qquad (2)$$

$\beta$ being a constant measuring the inhibitory effect of A. That is, if $\Delta I_s$ is zero and if $\Delta A$ is zero, then $\Delta S$ and S do not change. This would hold if the intake is not changed and if the hormone level is not changed from its previous level. But when $\Delta I_s$ is increased, i.e., increased load, the salt content increases, or if $\Delta A$ is increased, the salt content increases at a rate $\beta \Delta A$ because of the inhibition represented by the coefficient $\beta$. Considering only changes in A, equation (1) can be written, since the normal level $A_n = P/m$,

$$\frac{d\Delta A}{dt} = -\alpha \Delta S - m\Delta A. \qquad (3)$$

That is, if $\Delta S = \Delta A = 0$ so that S and A have their normal values, $\Delta A$ does not change and, hence, A remains at its normal value.

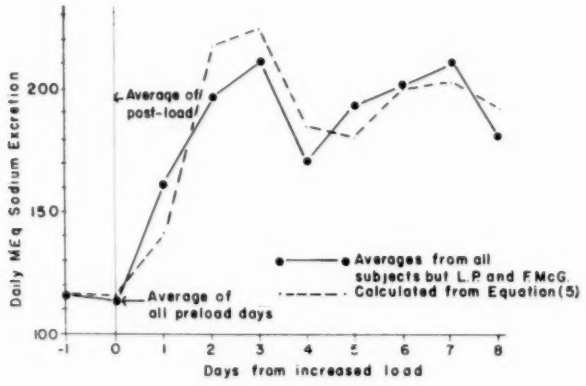


Fig. 5.

Now the excretion rate is $I_{sn} - \beta \Delta A$, the normal level of intake minus the amount inhibited (for $\Delta A$ positive). Hence, if we know $\Delta A$, we can calculate the excretion rate. The quantity $\Delta A$ can be obtained from (2) and (3) with the condition that when the intake is suddenly increased, the value of $\Delta A$ does not change immediately, i.e., $d(\Delta A)/dt = 0$ for $t = 0$. Then we can obtain the solution for the excretion rate as a function of time:

$$E_s = I_s - \Delta I_s e^{-\frac{1}{2}mt} \left(\frac{m}{2w} \sin wt + \cos wt\right) \qquad (4)$$

where $w = \sqrt{\alpha\beta - \frac{1}{4}m^2}$. Note that there are only two parameters, m and w.

Since it is necessary to collect daily samples, we may take the daily integral of (4) and call them E's. If $m = 0.66$ per day, $w = 1.55$ per day, $I_s = 196$, and $I_{sn} = 116$ mEq per day, then this leads to the following numerical equation for daily excretion in milliequivalents:

$$E'_s = 196 - e^{-0.33t} (17 \sin 89t + 80 \cos 89t), \qquad (5)$$

t being in days and the angles in (5) being in degrees instead of radians (1 radian = 57.3 degrees).

In Fig. 5, the average of all subjects except L. P. and F. McG. (higher salt load than others) are shown by the solid curve. The average of all preload excretion values is 116, whereas the average of all postload values is 196 (196 − 116 = 80).

Shown in the same figure are the calculated values from equation (5). It can be seen that the 2 curves show a considerable degree of similarity. It should be pointed out that since m = 0.66 per day, the half life of A is about 1 day for these data in terms of this model.

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# BACTERIAL INVASION OF BLOOD AFTER ORAL SURGICAL PROCEDURES

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THE incidence of bacterial invasion of the blood following oral surgical procedures has been studied by several investigators.[1-8] The likelihood of obtaining positive blood cultures in connection with these procedures appears to be greater when samples of blood are taken at the conclusion of the operation rather than 10 minutes later. Also, bacterial invasion of the blood stream may be decreased by administration of an antibiotic prior to oral surgery.[2, 3] Recommendations, designed to prevent bacterial invasion of the blood following dental surgery, were made by the American Council on Rheumatic Fever and Congenital Heart Disease of the American Heart Association.[9]

Investigations of the incidence of pathogenic organisms in the blood after oral surgical procedures at the Mayo Clinic had not been extensive. In the present study we have attempted to compare the incidence of bacterial invasion of the blood of patients undergoing such surgery without receiving any antibiotic beforehand with that of patients who were given phenoxymethyl penicillin[10, 11] by mouth prior to operation. We were interested in determining whether penicillin in this form without adjunctive parenteral administration of penicillin G would be an effective prophylactic. Also, we wished to know whether the agent, given 24 rather than 48 hours before operation, would suffice.

## PROCEDURE

Samples of blood for cultures were obtained from 127 patients immediately after oral surgical procedures which included extraction of teeth. Another sample was obtained 18 hours later. None of the patients had urinary obstruction or evidence of renal disease, and none had cardiovascular defects which would make them vulnerable to endocarditis or endarteritis. The 50 patients who will be referred to as constituting "group A" did not receive any antibiotic or chemotherapeutic agent before operation. The remaining 77 subjects received only 1 or 2 tablets of phenoxymethyl penicillin* (500,000 U. or 300 mg. per tablet) at intervals of approximately 6 hours for a total of 4 doses preceding the operation, which was undertaken about 4 hours after the last dose. Fifty of these 77 patients thus received 4,000,000 U. penicillin preoperatively and will be referred to as "group B." This amount of penicillin was chosen as approximating the preoperative dose recommended by the American Heart Association for patients with

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*Supplied as Pen-V, courtesy of Wyeth Laboratories, Philadelphia, Pa.

cardiovascular defects that would make them susceptible to bacterial endocarditis or endarteritis.[9] The American Heart Association recommended that a total dose of 3,200,000 to 4,200,000 U. penicillin be given prior to extraction of a tooth and that the larger amount be given in part by mouth and in part by injection. Also the schedule, as outlined by that association, recommends that treatment begin approximately 48 hours before operation. The schedule followed in this study appeared to be more applicable to the practice of oral surgery at the Mayo Clinic where it is sometimes difficult to begin treatment more than 24 hours before extraction.

The remaining 27 of the 77 patients (group C) received 1 rather than 2 tablets of phenoxymethyl penicillin every 6 hours for 4 doses, for a total preoperative dose of 2,000,000 U. of the antibiotic.

The presence of bacteria in the blood was determined by culturing approximately 10 ml. of blood in appropriate liquid and solid media. If the patient had received penicillin, penicillinase was added to the culture medium. Identification of a positive blood culture was made by the usual bacteriologic procedures.

The number and type of oral surgical procedures carried out on patients in the various groups are as follows: of the 50 patients in group A, 12 had more than 15 teeth extracted; 23 had 1 to 4 impacted teeth removed, and 1 had a torus palatinus excised. Of the 50 patients in group B, 10 had more than 15 teeth removed; 29 had impacted teeth removed, and 2 had large cysts excised. Of the 27 patients in group C, 4 had more than 15 teeth removed; 20 had impacted teeth removed, and 1 had a sequestrectomy for osteomyelitis.

## RESULTS

Twenty-seven of the 50 untreated patients (group A) had positive cultures of the blood obtained at operation immediately after oral procedure. Ten of these positive cultures grew *Streptococcus mitis* and one yielded *Staphylococcus aureus*. Eighteen hours after operation the blood of all of these 27 patients was negative for bacteria. However, organisms grew in cultures of the blood drawn 18 hours after operation from 5 other patients, even though cultures of the blood of these patients taken at operation had been negative (Table I).

TABLE I. BLOOD CULTURES FOR 127 PATIENTS AFTER ORAL OPERATIONS WITH AND WITHOUT PREOPERATIVELY ADMINISTERED PHENOXYMETHYL PENICILLIN

| | BLOOD DRAWN FOR CULTURES FROM | | | | | |
|---|---|---|---|---|---|---|
| | GROUP A (CONTROLS) | | GROUP B* | | GROUP C† | |
| | AT OPERATION | 18 HR. AFTER OPERATION | AT OPERATION | 18 HR. AFTER OPERATION | AT OPERATION | 18 HR. AFTER OPERATION |
| No. of patients in groups | 50 | | 50 | | 27 | |
| *Positive Cultures.*— | | | | | | |
| Streptococcus mitis | 10 | | | | 1 | |
| Streptococcus, nonhemolytic | 1 | | | | | |
| Staphylococcus aureus | 1 | 1 | | | | |
| Staphylococcus, coagulase-negative | 7 | 2 | 3 | 1 | 3 | 2 |
| Peptococcus | | 1 | | | | |
| Unidentified gram-negative bacillus | 1 | | | 1 | | |
| Corynebacterium | 7 | 1 | 5 | 1 | 1 | 2 |
| Total with positive cultures | 27 | 5 | 8 | 3 | 5 | 4 |
| Total with negative cultures | 23 | 45 | 42 | 47 | 22 | 23 |

*Received 4,000,000 U. phenoxymethyl penicillin prior to operation.
†Received 2,000,000 U. phenoxymethyl penicillin prior to operation.

Eight of the 50 patients comprising group B had positive cultures of blood drawn immediately after operation. Eighteen hours after operation the blood of all 8 was negative for bacteria. However, the blood drawn at 18 hours after operation from 3 other patients grew organisms even though cultures of blood of these 3 patients taken at operation had been negative. No streptococci were encountered in group B.

Five of the 27 patients in group C had positive cultures from the blood obtained immediately after operation. One of these cultures yielded *Str. mitis*. None of the 5 had positive cultures of blood drawn 18 hours after operation. Four additional patients in this group of 27, however, had positive cultures of blood drawn 18 hours after operation, even though the cultures made immediately after operation were negative.

For each patient a careful notation was made of the status of the oral hygiene, the duration of operation, and the magnitude of extraction. No correlation could be detected between these factors and the incidence of positive cultures.

## COMMENT

Our data appear to corroborate the findings of others, in that a significantly greater number of positive blood cultures occurred in the group of 50 patients who did not receive penicillin prior to oral surgery.

It is of interest that all patients with positive cultures of blood drawn immediately after operation had negative cultures of blood obtained 18 hours after the procedure. While this finding seems to indicate that bacterial invasion of the blood after oral surgical procedures is usually a transient phenomenon, it does not follow that such a phenomenon should be regarded lightly. Although some of the organisms isolated in this study are virtually never associated with primary infections, it is known that they can cause endocarditis. Previous studies[10, 12, 13] indicate that dental surgery can be causative in persistent bacteremia.

From our data it appears that phenoxymethyl penicillin is at least as effective in the dosage schedule employed as is parenterally administered penicillin G employed by other investigators. Rhoads and associates[5] administered 300,000 U. procaine penicillin G to 43 patients 2 hours prior to dental extraction and encountered postoperative bacterial invasion of the blood in 3 patients.

Glaser and others[6] studied 40 patients who had not received prophylactic treatment before operation and 40 who had received it. Streptococci were found on culture of the blood from more than 67.5 per cent of the patients in the first group. In the second group, in which the patients were given 600,000 U. penicillin intramuscularly in divided doses for 24 hours prior to dental extraction, the incidence of positive blood cultures was 42.5 per cent. In accord with our own data these authors found a marked decrease in the incidence of alpha hemolytic streptococci in the treated series.

Hirsh and others[7] administered 300,000 U. procaine penicillin G for 2 days prior to dental operation and stated that the incidence of bacterial invasion of the blood decreased from 46 to 37 per cent. When they considered

the alpha and gamma streptococci alone, they obtained a significant reduction in the incidence of transient bacteremia from 34 to 15 per cent. Like us these authors were unable to correlate the incidence of positive blood cultures with the state of oral hygiene or number of teeth extracted; however, they found a greater incidence of positive cultures after extensive surgical procedures.

McEntegart and Porterfield[3] found transient streptococcal bacteremia in 54 per cent of 200 patients immediately after dental extraction. They found no relationship to the extent of oral sepsis but only to the number of teeth removed and to the extent of the procedure.

Our data compare favorably with those of other workers since our 50 patients (group B) who received the higher total dose of phenoxymethyl penicillin (4,000,000 U.) demonstrated no growth of bacteria generally considered pathogenic for endocarditis in blood cultures obtained at the time of, and 18 hours after, oral operation. Our observations in group B suggest that when 4,000,000 U. phenoxymethyl penicillin is to be given by mouth this treatment need not begin until 24 hours before operation rather than 48 hours beforehand. Our observations indicate, too, that for prophylaxis, penicillin may be given in the phenoxymethyl form of the agent by the oral route.

We are currently investigating the use of phenoxymethyl penicillin in an attempt to prevent bacterial endocarditis and endarteritis after dental extractions in patients with congenital or acquired cardiovascular defects that would make them susceptible to such complications.

## SUMMARY

For 27 of the 50 patients who had not received an antibiotic, cultures of blood drawn immediately after oral surgical procedures yielded bacteria. Ten of these positive cultures grew streptococci of the mitis group, and 1 yielded *Staphylococcus aureus*. For 8 of a similar group of 50 patients who had received phenoxymethyl penicillin preoperatively every 6 hours for 4 doses totaling 4,000,000 U., cultures of blood drawn immediately after surgery grew bacteria; however, no streptococci of the mitis group were encountered. When half this dose, or 2,000,000 U., was used for 27 patients, cultures of blood immediately after surgery from 5 grew bacteria; the blood of one of these 5 yielded *Streptococcus mitis*.

Phenoxymethyl penicillin is effective in decreasing the incidence of bacterial invasion of the blood after oral surgery. When given in doses employed in this study, the agent seems to minimize invasion of the blood by streptococci of the mitis group. A total of 4,000,000 U. given preoperatively in 4 equally divided doses proved as effective as have other forms of penicillin given on differing schedules by others.

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# THE ESTIMATION OF FUCOSE IN SALIVA

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PURE human blood group active mucoids A, B, H, and Le[a] have been prepared and analyzed by Morgan and his collaborators.[1-4] These substances have been found to contain differing amounts of the methyl-pentose L-fucose. They are secreted in saliva.[5-7]

Precise methods are available for the estimation of methyl pentoses.[8, 9] This article shows that Gibbons' method[9] can be used to estimate the fucose content of human saliva.

## EXPERIMENTAL

*Materials.*—

*Salivas:* These were collected by having the subject spit in a test tube. The quantity collected varied from 2.5 to 5.0 ml. Artificial stimuli to salivation were not employed. The specimens were boiled for 10 minutes on a water bath immediately after collection, centrifuged at 2,000 r.p.m. for 5 minutes, and the supernatants were stored at −20° C.

*Red blood cells:* While the saliva sample was being collected, the subject's ear was punctured with a needle. A few drops of blood were collected in 2.0 ml. of physiologic saline. The red cells were "grouped" within 24 hours.

*L-fucose:* The pure sugar was heated *in vacuo* at 80° C. for 20 minutes and allowed to cool *in vacuo* over phosphorus pentoxide before it was used to make up the standard solution. The concentrated standard was kept at −20° C. when not in use.

*Thioglycollic acid:* A fresh solution (3.3 per cent v/v in distilled water) was prepared for each experiment.

*Sulfuric acid reagent:* Sulfuric acid (A.R.) 6 parts; distilled water 1 part.

*Antisera:* A saline extract of *Ulex europeus* seeds prepared by the method of Boyd and Shapleigh[10] was used as an anti-H agglutinin. Anti A and anti B were of the non-immune type from human sera. The same standardized solutions of the agglutinins were used throughout.

*Methods.*—The salivas were thawed at room temperature after removal from storage, then centrifuged for 10 minutes at 1,500 r.p.m. The supernatant was decanted and the small precipitate was discarded.

*Classification into secretor type:* All salivas were tested for H, A, and B activity quantitatively by means of a doubling dilutions agglutination inhibition technique.[11] Standard salivas from known blood group O, A, and B secretors were included in each day's estimations. The serologic end point taken was the greatest dilution which completely inhibited agglutination.

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*Red cell grouping:* This was carried out in the usual manner by means of a tube technique.

*Methyl pentose reaction of varying dilutions of saliva:* The same specimen of a blood group B secretor saliva was diluted to concentrations of ½, ¼, and ⅛ with distilled water. To 0.5 ml. of these concentrations, and to 0.5 ml. of the neat saliva, a 0.5 ml. of a 30.4 μg per milliliter solution of pure L-fucose in water was added. One half milliliter of water was added to a duplicate range of saliva dilutions. The methyl pentose content of all specimens was determined by Gibbons'[9] method.

*Recovery Experiment:* One-half milliliter of each of 9 different concentrations (range 5 to 45 μg per milliliter) of pure L-fucose in water was prepared in duplicate; 0.5 ml. of ¼ strength blood group B secretor saliva in water was added to each tube of one set of dilutions and 0.5 ml. of water to each tube of the other set. The methyl pentose content of all tubes was then determined by Gibbons' method.

The regressions of optical density upon fucose concentration for both lines, and the standard deviations of scatter about the lines were determined by the methods of Cornfield.[12] The 2 regression coefficients were compared by the method of Brownlee.[13]

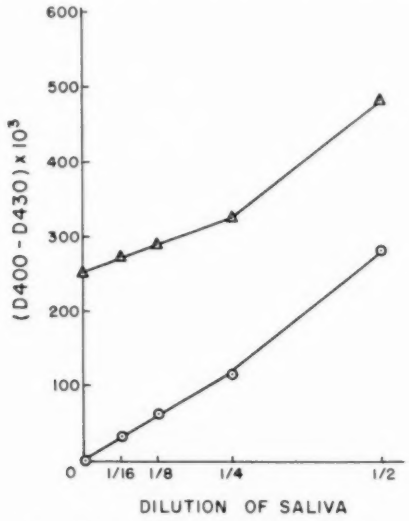


Fig. 1.—⊙ Saliva alone; △ added fucose 30.4 μg per milliliter. The relationship of different concentrations of saliva to the optical density produced in the thioglycollic acid test.

*Standard error of estimation of salivary fucose:* A large sample of blood group O nonsecretor saliva was divided into 25 portions. Each portion was treated as an individual saliva sample and its methyl pentose content determined by Gibbons' method.

*Procedure for methyl pentose estimation in saliva samples by a modification of Gibbons' method:* After centrifugation, 0.5 ml. of the saliva supernatant was diluted to 4.0 ml. with distilled water in a volumetric flask.

For each test the following tubes were prepared: (1) duplicate blanks which contained distilled water instead of the saliva solution, (2) five different concentrations of L-fucose (5 to 40 μg per milliliter) in duplicate as standards, and (3) the diluted saliva in triplicate. Thioglycollic acid reagent was added to 2 of the tubes only; the third served as a specimen blank.

After the sulfuric acid reagent had been added to all tubes at 0° C. the tubes were allowed to equilibrate with a warm room temperature of 26° C. This usually took 30 to 40 minutes; when it had taken place the tubes were boiled.

Readings were taken in a Hilger Uvispek spectrophotometer at 390, 395, 400, 405, and 430 mμ on all saliva specimens, and only at 400 and 430 mμ on the fucose standards and the water blanks. These readings were taken between 3 and 6 hours after the addition of thioglycollic acid. The readings on the standards were taken after one-half the saliva specimens had been read so that any error caused by the passage of time while the readings were taking place would be minimized.

## RESULTS

*Varying Dilutions of Saliva.*—When the same specimen of saliva was diluted to varying strengths, the results shown in Fig. 1 were obtained, with and without added L-fucose. It is seen that the relationship of optical density to concentration is practically linear up to ¼ strength of saliva.

*Recovery Experiment.*—The estimated regressions of optical density (y) expressed as (D400-D430) × $10^3$ (where D = optical density) on the final concentration of pure fucose (x) were as follows:

(1) $y = 151.90 - 16.180\ x$; Sr = 9.354 units of y
(2) $y = 2.37 - 16.354\ x$; Sr = 5.756 units of y

where Sr is the standard deviation of scatter.

*Contaminated Specimens.*—It was found with pure fucose solutions and with saliva obtained from subjects in the fasting state after they had cleaned their mouths, that D395 was greater than D400, and also that D390 was greater than D405.

With contaminated specimens—many of which gave extremely high values for D400-D430—these relationships did not obtain, indicating a different type of absorption curve with its peak sometimes at 400 mμ and occasionally at 405 mμ. Usually the blanks for these contaminated specimens were markedly pink, whereas for uncontaminated salivas the blanks were practically colorless. When subjects had recently drunk tea or coffee their salivas were found to be contaminated.

*Error of Estimation of Salivary Fucose.*—When a large sample of group O nonsecretor saliva was divided into 25 portions and each was treated as a separate sample, a mean value of 69.62 μg fucose per milliliter was obtained. The standard error of the mean was 0.55 μg per milliliter.

*Fucose Content of Saliva.*—The fucose content of a particular individual's saliva can vary considerably at different times. (Table I.)

## DISCUSSION

It is seen from the results shown in Fig. 1 that the relationship of optical density in Gibbons' methyl pentose test to the concentration of saliva is practically linear up to ¼ strength; this obtains for dilutions with and without added pure fucose solution.

These results and those obtained from the recovery experiment indicate that there is no interference by saliva at one-eighth strength in the fucose reaction. Over the range tested in the recovery experiment (5 to 45 μg fucose

TABLE I. VARIATIONS IN THE FUCOSE CONTENT OF AN INDIVIDUAL'S SALIVA

| SUBJECT | BLOOD GROUP | ABH SECRETOR STATUS | FUCOSE CONCENTRATIONS OF SPECIMENS OF SALIVA OBTAINED AT DIFFERENT TIMES (μg/ml.) | | | | |
|---|---|---|---|---|---|---|---|
| M | B | + | 54.0, | 66.7 | --- | --- | --- |
| F | O | + | 75.8, | 95.4, | 152.3 | --- | --- |
| E | B | + | 46.2, | 55.8, | 62.3, | 95.0, | 133.1 |
| C | O | 0 | 69.7, | 76.7 | --- | --- | --- |

+ = ABH secretor; 0 = ABH nonsecretor.

per milliliter in the reaction mixtures) the slopes are virtually the same for both regression lines. (When the slopes are compared by the method of Brownlee,[13] $t = 0.682$ with 18 degrees of freedom and $p > 0.10$.)

Saliva specimens whose absorption curves did not obey the relationships described above for pure fucose and uncontaminated saliva were felt to be so contaminated as to yield unreliable results. This was presumably because the absorption curve of the fucose present was submerged by adjacent absorption curves of some other substances.

The variation in an individual's salivary fucose content from time to time may well represent the dilution of a component of saliva which contains fucose by another component which contains much less fucose or no fucose at all. Unfortunately the saliva samples were too small to determine their fucose in terms of "solid content."

Blood group substances are known to contain from 12 to 20 per cent of fucose by weight,[1-4] and they are known to exist in saliva.[5-7] Small amounts of fucose are known to exist in other substances in the human body, e.g., intrinsic factor,[14] urinary mucoprotein, virus inhibitor,[15, 16] and oligosaccharides of human milk.[17] The quantities contained in these substances are small, and it is possible in some of them that the fucose is a contaminant, as these substances are obtained from liquids which possess blood group activity. These substances are not present in saliva. There are no methyl pentoses other than fucose in saliva.[18]

The results indicate that the fucose content of saliva can be accurately determined by subjecting a 1:8 aqueous dilution to Gibbons' method for methyl pentose. The estimation of salivary fucose content in this way may prove to be a reliable index of its blood group substance content.

## SUMMARY

Gibbons' method for methyl pentoses has been found to be a reliable method for the estimation of salivary fucose concentration. The standard error of measurement is of the order of 1 per cent.

A spectrophotometric technique is described for detecting salivas which are so contaminated as to prevent their fucose content being accurately determinable.

The salivary fucose content of a particular individual varies considerably from time to time.

Blood group substances are known to be present in considerable amounts in saliva, and these substances are known to contain 12 to 20 per cent by

weight of fucose. The estimation of salivary fucose content may prove to be a reliable index of blood group substance content.

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# THE FUCOSE AND AGGLUTINOGEN CONTENTS OF SALIVA IN SUBJECTS WITH DUODENAL ULCERS

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HUMAN individuals are divided into two types by the secretor phenomenon,[1] the ability to secrete A, B, and H blood group substances into the body liquids. The liquid which can be tested most easily to determine the individual's secretor status is the saliva. Substances A and B are specific products of the genes so named. Substance H is found in the salivas of blood group O, A, and B secretors and hence cannot be regarded as a specific gene product.[2] Individuals who do not secrete A, B, and H substances secrete Lewis blood group substance in their saliva.[3] There are a very few individuals who do not secrete any recognizable blood group substance in their salivas.[4] Secretors of A, B, and H substances also secrete Lewis substance in their saliva, but in very much smaller concentrations than ABH nonsecretors.

There are a significantly higher proportion of ABH nonsecretors in series of subjects with duodenal ulcers than are present in control series taken from the general population.[5, 6] This indicates that ABH nonsecretors have a greater susceptibility to develop duodenal ulcers than have ABH secretors. The suggestion has been made[5] that this increased susceptibility of ABH nonsecretors is due to the fact that their duodenal mucosae are protected by smaller concentrations of blood group substances than are the duodenal mucosae of ABH secretors.

The pure human blood group active mucoid substances A, B, H, and Le[a] have been isolated and analyzed by Morgan and his colleagues.[7-10] They all contain considerable amounts of the methyl pentose L-fucose. There are accurate methods available for estimating methyl pentose.[11, 12] One such method, that of Gibbons,[12] has been shown to be a reliable technique for estimating salivary fucose concentration.[17]

The aims of this article are: (1) to show that salivary fucose determinations provide a reliable index of total salivary blood group mucoid content, (2) to present evidence that ABH nonsecretors secrete similar concentrations of blood group mucoids in their salivas to ABH secretors, and (3) to show for 2 phenotypes that there is no significant difference between the total salivary blood group mucoid concentrations of subjects with duodenal ulcers and subjects without duodenal ulcers.

These findings cast doubt on the concept that there is less protection of the duodenal mucosa by blood group mucoids in ABH nonsecretors when compared with ABH secretors.

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## EXPERIMENTAL

*Materials.*—

*Salivas:* These were collected by having the subject spit in a test tube. The quantity collected varied from 2.5 to 5.0 ml. Artificial stimuli to salivation were not employed. The specimens were boiled for 10 minutes on a water bath immediately after collection, centrifuged at 2,000 r.p.m. for 5 minutes, and the supernatants were stored at −20° C.

*Red blood cells:* While the saliva sample was being collected, the subject's ear was punctured with a needle. A few drops of blood were collected in 2.0 ml. physiologic saline. The red cells were "grouped" within 24 hours.

*Antisera:* A saline extract of *Ulex europeus* seeds prepared by the method of Boyd and Shapleigh[13] was used as an anti-H agglutinin. Eel serum (from *Anguilla anguilla*[14]) was used as an anti-H agglutinin for one series of subjects in addition to the Ulex extract. Anti-A, anti-B, and anti-Le$^a$ agglutinins were of the nonimmune type from human sera. The same standardized solutions of the agglutinins were used throughout.

*L-fucose:* The pure sugar was heated *in vacuo* at 80° C. for 20 minutes and allowed to cool *in vacuo* over phosphorus pentoxide before it was used to make up the standard solution. The concentrated standard was kept at −20° C. when not in use.

*Thioglycollic acid:* A fresh solution (3.3 per cent v/v in distilled water) was prepared for each experiment.

*Sulfuric acid reagent:* Sulfuric acid (A.R.) 6 parts; distilled water 1 part.

*Methods.*—The salivas were thawed at room temperature after removal from storage, and were centrifuged for 10 minutes at 1,500 r.p.m. The supernatant was decanted and the small precipitate was discarded.

*Classification into secretor type:* All salivas were tested for H, A, and B activity quantitatively by means of a doubling dilutions agglutination-inhibition technique.[15] Standard salivas from known blood group O, A, and B secretors were included in each day's estimations. The serologic end point taken was the greatest dilution which completely inhibited agglutination. A series of blood group O, ABH nonsecretor salivas were quantitatively tested for Le$^a$ activity by means of the same doubling dilutions technique.

*Red cell grouping:* This was carried out in the usual manner by means of a tube technique.[16]

*Procedure for estimating fucose in saliva samples by Gibbons'[12] method:* This procedure was carried out as described in a previous paper.[17] Five tenths milliliter of saliva was diluted to 4.0 ml. with distilled water in a volumetric flask. Each saliva was then processed in triplicate. Thioglycollic acid was added to two tubes and the third served as a specimen blank. Spectrophotometric readings of optical density (D) were made on each specimen at 5 wavelengths, viz.: 390, 395, 400, 405, and 430 mμ. A series of 5 standard fucose concentrations (5 to 40 μg per milliliter) in duplicate, and 2 water blanks were included with every batch of saliva specimens. The regression equation, $y = a + bx$ (where $y = (D_{400} - D_{430}) \times 10^3$, and $x$ = concentration of fucose in μg per milliliter), was calculated for the standards in each experiment. The fucose concentrations of the salivas were calculated from the regression equation.

*Statistical techniques:* The $\log_{10}$ salivary fucose concentrations in the different nonsecretor phenotypes have been subjected to a one-way analysis of variance procedure.[18]

The means of the $\log_{10}$ salivary fucose concentrations of the different phenotypes have been compared by the procedure of Scheffé[19] which circumvents the difficulties of multiple testing.

The confidence limits of a difference as determined by this method are:

$$\pm \sqrt{(k-1)\ F_{\alpha\ (k-1)n_2} \left( \frac{MS_{wp}}{N_1} + \frac{MS_{wp}}{N_2} \right)}$$

where k = number of phenotypes, F is obtained from variance ratio tables at $\alpha$ probability, $n_2$ = degrees of freedom within phenotypes, $MS_{wp}$ = mean square within phenotypes, and $N_1$ and $N_2$ = numbers of observations in the two phenotypic classes compared. When there is a difference at the level of significance indicated, the confidence limits do not overlap zero.

The regressions, $y = a + bx$ of the $\log_{10}$ salivary fucose concentrations (y) upon the agglutinogen titers of the salivas (x), have been calculated. For this purpose the last tube which completely inhibited agglutination was taken as the measure of agglutinogen concentration and was expressed as follows: $\frac{1}{2} = 1$, $\frac{1}{16} = 4$, $\frac{1}{64} = 6$, etc. This is effectively a logarithmic scale.

The slopes of the regression equations are compared by the method of Brownlee.[20]

## RESULTS

*Contaminated Specimens.*—Results from saliva specimens for which D400 was greater than D395, and D405 was greater than D390, were considered unreliable.[17] For this reason the results from 163 samples had to be discarded.

*Error of Estimation of Salivary Fucose.*—The salivary fucose concentrations presented in this article were obtained in 30 experiments. The mean values of the parameters for the 30 standard lines are shown in Table I. The mean standard error of x calculated from the value of y observed on a single sample is 0.66 $\mu$g per milliliter fucose.

TABLE I. PARAMETERS FOR 30 "STANDARD LINES" WHICH EXPRESS THE RELATIONSHIP BETWEEN OPTICAL DENSITY (y) EXPRESSED AS (D400-D430) × $10^3$, AND FUCOSE CONCENTRATION ($\mu$g/ml. x)

| ESTIMATED PARAMETER | MEAN | STANDARD DEVIATION OF THE DISTRIBUTION |
|---|---|---|
| Regression Coefficient b | 16.7139 | 0.5410 |
| Intercept a (in y units) | −3.3421 | 3.0370 |
| Standard Deviation of scatter $S_r$ (in y units) | 11.0172 | 4.8210 |
| Standard Error of x calculated from a single observation of y (units of x) | 0.6585 | 0.2885 |

TABLE II. MEAN $\log_{10}$ SALIVARY FUCOSE CONCENTRATIONS OF SUBJECTS WITHOUT ULCERS

| BLOOD GROUP | ABH SECRETOR STATUS | NUMBER EXAMINED | MEAN $\log_{10}$ FUCOSE CONCENTRATION (M) | STANDARD ERROR OF THE MEAN (M) | ANTILOG OF THE MEAN (M) ($\mu$G/ML. FUCOSE) |
|---|---|---|---|---|---|
| O | + | 94 | 1.8982 | 0.0183 | 79.11 |
| A | + | 62 | 1.9250 | 0.0212 | 84.14 |
| B | + | 57 | 1.9759 | 0.0269 | 94.61 |
| All | + | 213 | 1.9268 | 0.0126 | 84.48 |
| O | − | 57 | 1.8100 | 0.0273 | 64.57 |
| A | − | 53 | 1.8051 | 0.0253 | 63.84 |
| B | − | 9 | 1.7535 | 0.0384 | 56.59 |
| All | − | 119 | 1.8035 | 0.0174 | 63.60 |

+ = secretor; − = nonsecretor.

TABLE III. MEAN $\log_{10}$ SALIVARY FUCOSE CONCENTRATIONS OF SUBJECTS WITH DUODENAL ULCERS

| BLOOD GROUP | ABH SECRETOR STATUS | NUMBER EXAMINED | MEAN $\log_{10}$ FUCOSE CONCENTRATION (M) | STANDARD ERROR OF THE MEAN (M) | ANTILOG OF THE MEAN (M) ($\mu$G/ML. FUCOSE) |
|---|---|---|---|---|---|
| O | + | 90 | 1.9307 | 0.0226 | 85.25 |
| O | − | 50 | 1.7388 | 0.0366 | 54.80 |

+ = secretor; − = nonsecretor.

*Subjects Without Ulcers.*—The distributions of $\log_{10}$ salivary fucose concentrations approximate normal curves, whereas the crude values do not. The $\log_{10}$ values are, therefore, used for computations; their distributions are shown in Fig. 1. The numerical data are given in Table II.

The salivas of blood groups O and A nonsecretors of ABH in Table II were all tested with anti-$Le^a$ antibody. All salivas inhibited anti-$Le^a$ in the group O nonsecretor category. In the blood group A nonsecretor category there were 3 salivas which gave very weak anti-$Le^a$ inhibition, but none completely failed to inhibit anti-$Le^a$.

The titer levels of the agglutinogens of these 332 salivas are shown in Fig. 2.

*Subjects With Duodenal Ulcers.*—Salivas from subjects in two phenotypic classes (blood group O secretors of ABH, and blood group O nonsecretors of ABH) with proven duodenal ulcers were studied. The salivary agglutinogen titers of the blood group O secretors of ABH are shown in Fig. 3. The $\log_{10}$ fucose concentrations of the salivas are shown in Fig. 4 and Table III.

*Statistical Analysis.*—The $\log_{10}$ salivary fucose concentrations in the different phenotypes without ulcers have been subjected to an analysis of variance. The result is shown in Table IV. There is a significantly greater variation between phenotypes than there is within phenotypes.

The means of the different phenotypes are compared according to the procedure of Scheffé.[19] These comparisons are shown in Table V. It is seen

TABLE IV. ANALYSIS OF VARIANCE ON $\text{LOG}_{10}$ SALIVARY FUCOSE CONCENTRATIONS OF SUBJECTS WITHOUT ULCERS

| SOURCE OF VARIATION | DEGREES OF FREEDOM | SUMS OF SQUARES | MEAN SQUARE |
|---|---|---|---|
| Between phenotypes | 5 | 1.3984 | 0.27968 |
| Within phenotypes | 326 | 11.1914 | 0.03432 |

$$F = \frac{0.27968}{0.03432} = 8.149;\ p < 0.001.$$

TABLE V. COMPARISONS OF MEANS OF $\text{LOG}_{10}$ SALIVARY FUCOSE CONCENTRATIONS OF SUBJECTS WITHOUT ULCERS

| PHENOTYPES COMPARED | P | DIFFERENCE IN MEANS | CONFIDENCE LIMITS OF THE DIFFERENCE AT THE STATED LEVEL OF P | |
|---|---|---|---|---|
| | | | LOWER | UPPER |
| O + and O − | 0.05 | 0.0882 | −0.01496 | +0.19136 |
| *O + and all − | 0.05 | 0.0947 | +0.00965 | +0.17975 |
| †O + and all − | 0.01 | 0.0947 | −0.0046 | +0.1940 |
| *A + and O − | 0.05 | 0.1150 | +0.00222 | +0.22778 |
| †A + and O − | 0.01 | 0.1150 | −0.0167 | +0.2467 |
| †A + and all − | 0.01 | 0.1215 | +0.0091 | +0.2339 |
| ‡B + and all − | 0.001 | 0.1215 | −0.0099 | +0.2529 |
| ‡B + and O − | 0.001 | 0.1659 | +0.0088 | +0.3230 |
| ‡H + and all − | 0.001 | 0.1724 | +0.0373 | +0.3075 |
| ‡AB + and all − | 0.001 | 0.1233 | +0.0273 | +0.2193 |

+ = ABH secretor; − = ABH nonsecretor.
Where the confidence limits do not include zero the difference is significant at the level of P stated.
*Significant at p = 0.05.
†Significant at p = 0.01.
‡Significant at p = 0.001.

that the means of the $\log_{10}$ salivary fucose concentrations of the blood groups A and B secretors are significantly different from the highest mean among the ABH nonsecretors, that of the blood group O nonsecretors. The mean $\log_{10}$

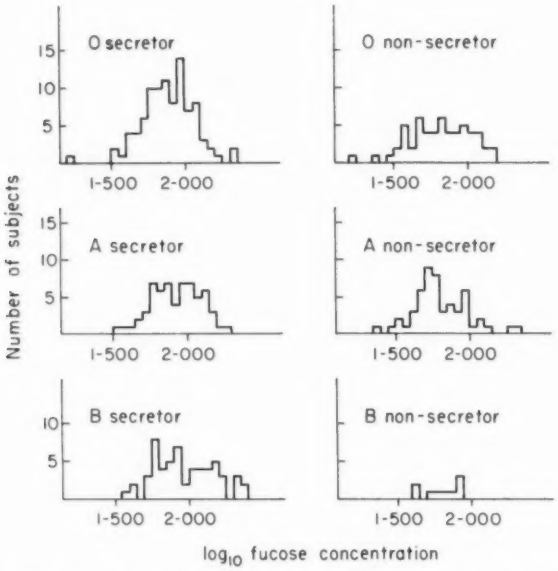


Fig. 1.—The distribution of $\log_{10}$ salivary fucose concentrations in subjects without ulcers.

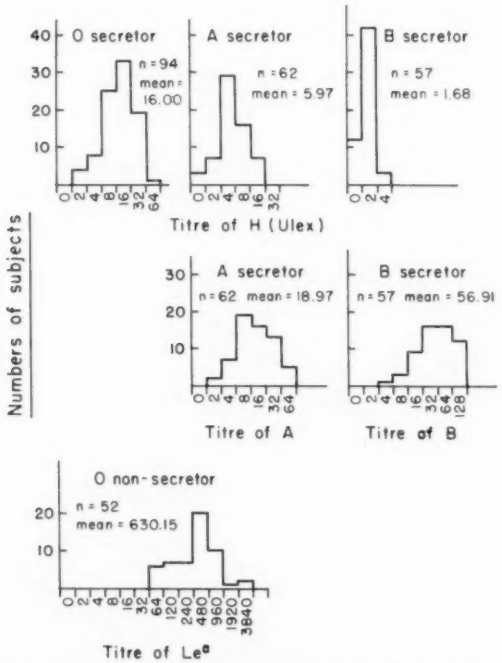


Fig. 2.—Agglutination-inhibition titers in subjects without ulcers.

salivary fucose concentration of the blood group O secretors is not significantly different from that of the blood group O nonsecretors, but is significantly higher than the mean for all the ABH nonsecretors combined. There is a highly significant difference between the mean $\log_{10}$ salivary fucose concentration of all ABH secretors combined and the mean for all ABH nonsecretors combined.

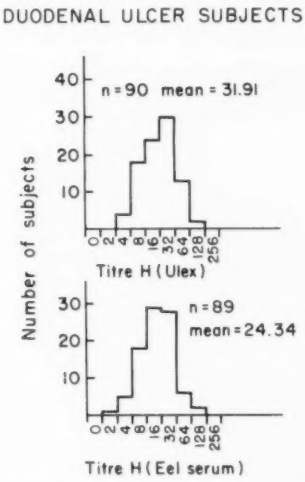


Fig. 3.—Agglutination-inhibition titers in blood group O secretor subject with duodenal ulcers.

The $\log_{10}$ salivary fucose concentrations of the subjects with duodenal ulcers and the corresponding subjects without ulcers were examined by the analysis of variance. The result is shown in Table VI; again, there is more variation between phenotypes than within phenotypes. The mean $\log_{10}$ salivary fucose concentrations of the different phenotypes are compared by Scheffé's method[19] in Table VII. It will be seen (1) that both series of blood group O secretors have mean $\log_{10}$ salivary fucose concentrations which are not sig-

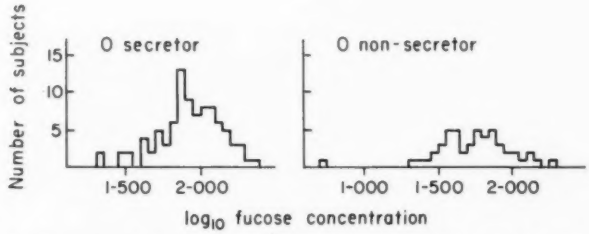


Fig. 4.—The distribution of $\log_{10}$ salivary fucose concentrations in subjects with duodenal ulcers.

nificantly different, (2) that both series of blood group O nonsecretors have mean $\log_{10}$ salivary fucose concentrations which are not significantly different, and (3) that the blood group O nonsecretors with duodenal ulcers have a mean $\log_{10}$ salivary fucose concentration which is significantly different from the means of both series of blood group O secretors.

The parameters of the estimated regressions of $\log_{10}$ salivary fucose concentrations upon the agglutinogen titers of salivas are shown in Table VIII. There is a significant correlation between all agglutinogens and $\log_{10}$ salivary

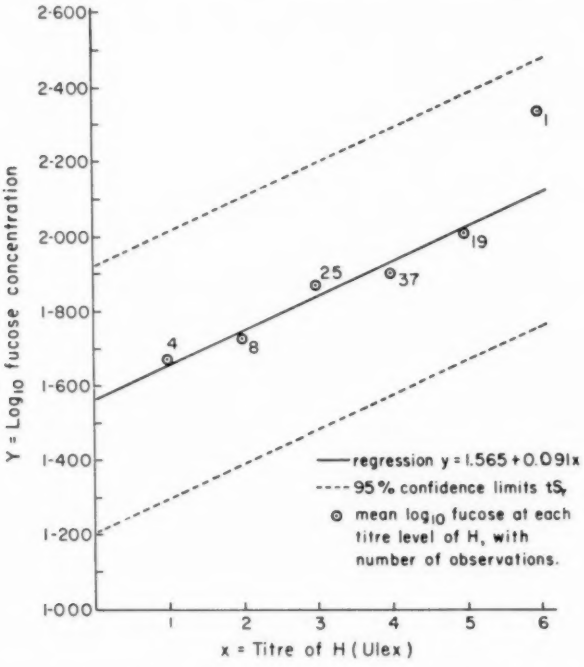


Fig. 5.—The regression of $\log_{10}$ salivary fucose concentration on agglutination-inhibition titer of H in blood group O secretor subjects without ulcers. ($S_Y$ is the standard deviation of scatter of the points about the line.)

fucose concentrations except in the case of A agglutinogen. Fig. 5 shows (as an example) the relationship between Ulex inhibiting titers and $\log_{10}$ salivary fucose concentrations in blood group O secretor individuals without ulcers.

## DISCUSSION

The regressions obtained between $\log_{10}$ salivary fucose concentrations and agglutinogen titers indicate that fucose estimation can be used to determine total blood group mucoid content of saliva.

The wide confidence limits of the regressions (Table VIII) are considered to be an expression of the variability of the serologic technique. A reason for the absence of a significant regression between $\log_{10}$ fucose concentration and

TABLE VI. ANALYSIS OF VARIANCE ON $\log_{10}$ SALIVARY FUCOSE CONCENTRATIONS OF GROUP O SECRETORS AND NONSECRETORS, WITH AND WITHOUT DUODENAL ULCERS

| SOURCE OF VARIATION | DEGREES OF FREEDOM | SUMS OF SQUARES | MEAN SQUARE |
|---|---|---|---|
| Between phenotypes | 3 | 1.4597 | 0.48656 |
| Within phenotypes | 287 | 12.7073 | 0.04427 |

$$F \text{ ratio} = \frac{0.48656}{0.04427} = 10.991;\ p < 0.001.$$

A titer can only be conjectured, but, possibly, it is due to the fact that blood group $A_1$ and $A_2$ persons were not differentiated. There are differences in the proportions of $Le^a$, H, and A substances secreted by these subtypes[21, 22] which may be responsible. On the other hand, this explanation would seem incompatible with the presence of a significant regression between $\log_{10}$ salivary fucose concentration and H titer in blood group A secretors.

It is suggested that the large variance of the salivary fucose concentration within a phenotypic class is due to the fact that a relatively high fucose secretion is diluted by one which contains a much smaller amount of fucose or no fucose at all. The fucose content in percentage of dry weight in saliva from 7 subjects has been determined.[23] This also shows a variation, the values ranging between 2.1 and 4.9.

The results presented here show that ABH secretors have a higher mean salivary fucose concentration than that of ABH nonsecretors. The difference is, however, not large. This finding must be assessed in the light of serologic knowledge and the known chemical structure of the group specific mucoids.

Nonsecretors of ABH secrete more $Le^a$ substance in their salivas than do ABH secretors[4]; in the various ABH secretors the relative proportions of A, B, H, and $Le^a$ substances are different. The approximate proportions are indicated in Table IX (for the sake of simplicity $Le^b$ has been ignored). Table X shows the fucose contents and molecular weights of pure human blood group active mucoids obtained from ovarian cyst fluids.[21] The fucose content of micromolar solutions is larger for A, B, and H substances than for $Le^a$ substance.

The salient facts which are known from serologic studies, coupled with knowledge of the properties of pure group specific mucoids and the salivary fucose contents of different phenotypes, allow one to formulate a concept of the quantitative aspects of blood group agglutinogen secretion. (Table XI.) The total salivary blood group mucoid has been computed for the different phenotypes by ascribing proportions of the mean $\log_{10}$ salivary fucose concentration to the various blood group mucoids as follows:

TABLE VII. COMPARISONS OF MEANS OF $\text{LOG}_{10}$ SALIVARY FUCOSE CONCENTRATIONS OF GROUP O SECRETORS AND NONSECRETORS, WITH AND WITHOUT DUODENAL ULCERS

| PHENOTYPES COMPARED | P | DIFFERENCE IN MEANS | CONFIDENCE LIMITS OF THE DIFFERENCE AT THE STATED LEVEL OF P | |
|---|---|---|---|---|
| | | | LOWER | UPPER |
| DU O+ and DU O– | 0.001 | 0.1919 | +0.0425 | +0.3412 |
| O+ and non DU O+ | 0.050 | 0.0325 | –0.0542 | +0.1192 |
| O+ and non DU O– | 0.010 | 0.1207 | +0.0004 | +0.2410 |
| O+ and non DU O– | 0.001 | 0.1207 | –0.0227 | +0.2641 |
| O– and non DU O+ | 0.001 | 0.1594 | +0.0112 | +0.3076 |
| O– and non DU O– | 0.050 | 0.0712 | –0.0426 | +0.1850 |
| DU O+ and non DU O– | 0.050 | 0.0882 | –0.0104 | +0.1868 |

+ = ABH secretor; – = ABH nonsecretor.

Where the confidence limits do not include zero the difference is significant at the level of P stated.

*Significant at 0.001.

†Significant at 0.01.

TABLE VIII. THE REGRESSIONS OF $LOG_{10}$ SALIVARY FUCOSE CONCENTRATIONS ON AGGLUTINATION-INHIBITION TITERS

| BLOOD GROUP | ABH SECRETOR STATUS | AGGLUTINOGEN EXAMINED | TYPE OF ANTIBODY | NUMBER OF SALIVAS EXAMINED | CORRELATION COEFFICIENT (R) | SIGNIFICANCE OF R | REGRESSION COEFFICIENT (B) | INTERCEPT (A) AND ITS ANTILOG ($\mu$G/ML. FUCOSE) | STANDARD DEVIATION OF SCATTER ($S_r$) (IN UNITS OF Y) |
|---|---|---|---|---|---|---|---|---|---|
| *Subjects Without Ulcers.—* | | | | | | | | | |
| O | + | H | Ulex | 94 | 0.5454 | $p < 0.001$ | 0.0911 | 1.5648 (36.71) | 0.1786 |
| A | + | A | Human non-immune | 62 | 0.1483 | $p > 0.10$ | 0.0200 | 1.8503 (70.84) | 0.1665 |
| A | + | H | Ulex | 62 | 0.5350 | $p < 0.001$ | 0.0938 | 1.7118 (51.50) | 0.1422 |
| B | + | B | Human non-immune | 57 | 0.2807 | $p < 0.05$ | 0.0462 | 1.7271 (53.34) | 0.1969 |
| B | + | H | Ulex | 57 | 0.3309 | $p < 0.02$ | 0.1367 | 1.8608 (72.58) | 0.1936 |
| O | − | $Le^a$ | Human non-immune | 52 | 0.3713 | $p < 0.01$ | 0.0539 | 1.3523 (22.51) | 0.2005 |
| *Subjects With Duodenal Ulcers.—* | | | | | | | | | |
| O | + | H | Ulex | 90 | 0.4993 | $p < 0.001$ | 0.1030 | 1.4524 (28.34) | 0.1873 |
| O | + | H | Eel | 89 | 0.4961 | $p < 0.001$ | 0.0937 | 1.5382 (34.53) | 0.1882 |

+ = secretor; − = nonsecretor.

(1) Nonsecretors of ABH are presumed to secrete Lewis substance only. This is all assumed to be $Le^a$.

(2) H titer and $\log_{10}$ salivary fucose content are correlated in blood group O secretors (Fig. 5 and Table IX). The intercept of the regression line is taken to represent fucose caused by the Lewis substance present. This (when antilogged) is 36.7 $\mu$g per milliliter. The fucose content of the Lewis substance is taken to be that of $Le^a$, i.e., 12 per cent.[21]

(3) Since fucose correlates with H titer in all ABH secretors, the ratios of fucose due to H substance in the various ABH secretors are taken to be the same as the ratios of the means of the anti-H (Ulex) inhibiting powers, viz.:

$$\text{B sec} : \text{A sec} : \text{O sec} :: 1.68 : 5.97 : 16.00$$

The fucose content due to H in blood group O secretors has been calculated as 42.4 $\mu$g per milliliter. (Antilog of the mean $\log_{10}$ salivary fucose concentration of nonduodenal ulcer blood group O secretors, minus the antilog of the intercept of the $\log_{10}$ salivary fucose-Ulex titer regression.) Therefore, the fucose caused by H in blood group A secretors is $\frac{42.4 \times 5.97}{16.00} = 15.8$ and in blood group B secretors, $\frac{42.4 \times 1.68}{16.00} = 4.4$ $\mu$g per milliliter.

(4) Grubb[4] showed that the approximate ratio of $Le^a$ activity in ABH secretors was:

$$(A + O) : (B + AB) :: 3 : 1$$

It is assumed that blood group O secretors and blood group A secretors have equal amounts of Lewis substance in their salivas. Since 36.7 $\mu$g per milliliter was held to be accounted for by Lewis substance in blood group O secretors the same amount is held to be present in blood group A secretors. In blood

TABLE IX. SEROLOGICALLY DETERMINED INFORMATION CONCERNING BLOOD GROUP ANTIGEN SECRETION[15]

| BLOOD GROUP | ABH SECRETOR STATUS | BLOOD GROUP MUCOID SECRETED | | | |
|---|---|---|---|---|---|
| | | B | A | H | LEWIS |
| B | + | +++ | − | + | + |
| A | + | − | +++ | ++ | ++ |
| O | + | − | − | +++ | ++ |
| All | − | − | − | − | +++ |

+ = secretor; − = nonsecretor.

TABLE X. THE MOLECULAR WEIGHTS AND FUCOSE CONTENTS OF PURE BLOOD GROUP ACTIVE MUCOIDS OBTAINED FROM OVARIAN CYST FLUIDS[19]

| SUBSTANCE | MOLECULAR WEIGHT | FUCOSE CONTENT (%) | $\frac{\text{MOLECULAR WEIGHT} \times \text{FUCOSE CONTENT}}{1 \times 10^5}$ (= FUCOSE CONTENT OF MICROMOLAR SOLUTION IN $\mu$G/ML.) |
|---|---|---|---|
| B | All | 18 | 45-54 |
| A | 250,000 | 18 | 45-54 |
| H | to | 20 | 50-60 |
| $Le^a$ | 300,000 | 12 | 30-36 |

TABLE XI. CALCULATION OF TOTAL BLOOD GROUP SUBSTANCE CONCENTRATION OF SALIVA

| BLOOD GROUP | ABH SECRETOR STATUS | ANTILOG MEAN $LOG_{10}$ SALIVARY FUCOSE ($\mu$G/ML.) | SUBDIVISION OF SALIVARY FUCOSE CONTENT | FUCOSE CONTENT OF PURE GROUP SPECIFIC MUCOID (%) | BLOOD GROUP SUBSTANCE CONCENTRATION ($\mu$G/ML.) | TOTAL SALIVARY BLOOD GROUP SUBSTANCE CONCENTRATION ($\mu$G/ML.) |
|---|---|---|---|---|---|---|
| All | – | 63.6 | All assumed to be $Le^a$ | 12 | $\frac{63.6 \times 100}{12} = 530.0$ | 530 |
| O | + | 79.1 | 36.7 $Le^a$ | 12 | $\frac{36.7 \times 100}{12} = 305.8$ | 518 |
| | | | 42.4 H | 20 | $\frac{42.4 \times 100}{20} = 212.0$ | |
| A | + | 84.1 | 36.7 $Le^a$ | 12 | $\frac{36.7 \times 100}{12} = 305.8$ | |
| | | | $\frac{42.4 \times 5.97}{16.00} = 15.8$ H | 20 | $\frac{15.8 \times 100}{20} = 79.0$ | 560 |
| | | | 31.6 A | 18 | $\frac{31.6 \times 100}{18} = 175.5$ | |
| B | + | 94.6 | $\frac{36.7}{3} = 12.2$ $Le^a$ | 12 | $\frac{12.2 \times 100}{12} = 101.7$ | |
| | | | $\frac{42.4 \times 1.68}{16.00} = 4.4$ H | 20 | $\frac{4.4 \times 100}{20} = 22.0$ | 557 |
| | | | 78.0 B | 18 | $\frac{78.0 \times 100}{18} = 433.7$ | |

+ = secretor; – = nonsecretor.

group B secretors the Lewis substance is held to account for $\frac{36.7}{3} = 12.2$ $\mu$g per milliliter fucose. This Lewis substance is assumed to have the same fucose content as $Le^a$, viz., 12 per cent.

(5) The following considerations have been ignored in compiling Table XI: (1) the occurrence of Lewis nonsecretor persons in both ABH secretor and ABH nonsecretor classes, and (2) $Le^b$ secretion. This is because there is at present insufficient information to incorporate these features into the scheme.

It is true that the estimated proportions of different blood group substances present in each type of individual, used in Table XI, is only an approximation but a radical change in the proportion ascribed would be required to influence materially the figure computed for total blood group substance. So great a change would seem to be inconsistent with independent serologic findings (Table IX).

This view of blood group secretion indicates that all phenotypes have roughly the same mean total concentration of blood group substances present in their salivas. Thus it seems likely that the greater susceptibility of ABH nonsecretors to duodenal ulceration as compared with that of ABH secretors is not caused by the lower concentrations of blood group substances in their body fluids.

The mean $\log_{10}$ salivary fucose concentration (and hence the mean total blood group substance concentration) seems to be the same in subjects with duodenal ulcers as it is in subjects without ulcers. This conclusion is strengthened by the fact that when the slopes of the regressions of $\log_{10}$ salivary fucose concentration upon Ulex inhibiting titer are compared in blood group O secretors without ulcers and group O secretors with duodenal ulcers, $t = 0.453$ and $p > 0.10$. When the intercepts of these two regressions are compared, $t = 1.054$ and $p > 0.10$. Hence there is no significant difference in the $\log_{10}$ salivary fucose concentration-agglutinogen titer relationship between subjects with and without duodenal ulcers.

The finding, that the mean total salivary blood group substance concentration is the same in subjects with duodenal ulcers as it is in subjects without ulcers, indicates that persons who have developed duodenal ulcers have not done so because they have lower total concentrations of blood group substances in their body liquids than have subjects without ulcers. There is no information available on the output of blood group substance per unit time at any site. It has been suggested[24] that blood group substances protect the duodenal mucosa against the development of ulcers. In view of the known increase in acid output from the stomach of subjects with duodenal ulcers[25] it would be of value to assess the gastric blood group substance output in subjects with this condition.

## SUMMARY

The concentration of L-fucose in saliva has been determined in 472 subjects, 332 of whom were free of peptic ulcer symptoms and 140 of whom had duodenal ulcers. The ABO blood group and ABH secretor status of these subjects was determined.

It has been shown that there is a significant correlation between $\log_{10}$ salivary fucose concentration and salivary agglutination-inhibition titer for the following agglutinogens: H in blood group A, B, and O secretors, B in blood group B secretors, and $Le^a$ in blood group O nonsecretors. The correlation between $\log_{10}$ salivary fucose concentration and salivary agglutinogen titer of H in blood group O secretors held true when both Ulex extract and eel serum were used as antisera. Significant correlation was absent between $\log_{10}$ salivary fucose concentration and salivary A agglutinogen titer in blood group A secretors.

The estimation of fucose in saliva is accurate to approximately 1 per cent. In view of the correlations found between salivary agglutinogen titer and salivary fucose concentration, it would appear that the latter is an accurate index of the total salivary blood group substance concentration.

Nonsecretors of ABH substances have been found to have almost as high a salivary fucose concentration as do ABH secretors. It has been computed that the mean total blood group substance concentration is of the same order, regardless of blood group or ABH secretor status.

ABH secretor and ABH nonsecretor blood group O subjects with duodenal ulcers have the same salivary fucose concentrations as the corresponding phenotypic classes without ulcers.

These findings suggest that the increased susceptibility of ABH nonsecretors to develop duodenal ulcers, when compared with ABH secretors, is not caused by the ABH nonsecretors having lower total blood group substance concentrations in their body liquids. The results further suggest that subjects who have developed duodenal ulcers have not done so because their body liquids (as exemplified by saliva) have lower total blood group substance concentrations than do those of subjects without ulcers.

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## ADDENDUM

Specimens of saliva from one subject (which are not included in any of the above series) repeatedly failed to exhibit any inhibition of anti-H, anti-A, anti-B, and anti-$Le^a$ activity. This person's saliva was found to contain 35.7 $\mu$g per milliliter fucose. Other ABH nonsecretor salivas which have fucose concentrations of this order possess $Le^a$ activity. There may be, therefore, a hitherto unrecognized fucose-containing mucoid in some salivas.

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# A COMPARATIVE STUDY OF RENAL CONCENTRATING ABILITY IN CHILDREN WITH SICKLE CELL ANEMIA AND IN NORMAL CHILDREN

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IT HAS been established by the studies of several investigators[1-4] that individuals with sickle cell anemia have a defect in renal concentrating ability when evaluated by measurement of the maximal urinary osmotic concentration (or maximal urinary specific gravity) attained during antidiuresis. The purpose of this study was to appraise the concentrating ability in children with sickle cell anemia by utilization of a second index of renal concentrating power, namely $T^c_m H_2O$,[5] which is the maximal rate of solute free water reabsorption achieved during osmotic diuresis. We have concluded from this investigation that these 2 parameters are not similarly affected by the pathophysiologic factors at work in children with this disease.

After this investigation was begun a similar study was reported by Heinemann and Cheung[6] which involved 4 subjects with sickle cell anemia. In only 1 child was $T^c_m H_2O$ determined, and the value obtained, 11.5 ml. per minute per 1.73 sq. M., would not permit an interpretation of this function in children, as the level was unaccountably high.

In this report data will be presented relative to $T^c_m H_2O$ in normal children, a function not previously evaluated in this age group.

## METHODS

The primary subjects were 16 children with sickle cell anemia diagnosed by the presence of anemia, reticulocytosis, and the typical S-hemoglobin electrophoretic pattern, supported by the presence of sickle cell hemoglobin in some form (sickle cell trait, sickle cell anemia, etc.) in both parents when available. Only 1 child (Subject B. J.) had received a transfusion within 6 months of this study. Twelve normal children were studied, either on admission to the hospital for elective minor surgery or after at least a 5 day convalescence at the hospital following a respiratory infection. All had a normal level of hemoglobin and demonstrated only A-hemoglobin by electrophoresis. Additional subjects were 3 children with sickle cell–hemoglobin-C disease and 1 child who is presumed to have sickle cell–thalassemia disease.

Urine was collected via an indwelling catheter with the bladder completely emptied by instilling air and expressing it by manual suprapubic pressure. Blood samples were

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collected from a convenient vein with an indwelling needle; their patency was maintained by injection of small quantities of heparin.

Food and liquids were withheld for 13 to 16 hours, usually 15 hours, from all subjects in preparation for study. After the bladder was emptied and hydropenic urine and blood samples were obtained, one or two 20 minute urine samples were collected to measure urine flow and water reabsorption in subjects in the oliguric state. Then, progressively increasing osmotic diuresis was induced by the intravenous infusion of 10 per cent mannitol in water at the rate of 20 ml. per minute per 1.73 sq. M., utilizing a constant infusion pump (Sigmamotor). Our objective was to obtain a urine flow of approximately 20 ml. per minute per 1.73 sq. M. in each subject. However, because the hyponatremia that ensues in individuals undergoing progressive osmotic diuresis is occasionally severe, in some of the younger children, the infusion was prophylactically discontinued before this level of urine flow was reached.

For the determination of $T_m^c H_2O$, urine was collected for 5 to 8 periods of 10 minutes each, with blood samples obtained 2 minutes after the onset of the period. In some subjects the first period was not begun until 20 to 30 minutes after initiation of the constant infusion. Therefore, the smaller value in Column 12, Tables I and II, does not represent the minimal urine flow at which $T_m^c H_2O$ was reached in all subjects.

Inulin clearance ($C_{IN}$) and renal plasma flow (RPF) were measured in some subjects, in which case a priming dose of inulin and sodium paraaminohippurate was injected after the oliguric urine and blood specimens had been obtained, and a sustaining dose of inulin and sodium paraaminohippurate was added to the 10 per cent mannitol. The priming and sustaining doses were calculated to achieve and maintain inulin and paraaminohippurate levels of approximately 20 mg. per cent and 1 to 2 mg. per cent, respectively. At least a 30 minute period for equilibration was allowed before inulin clearance and renal plasma flow were measured, thus these determinations were made during the course of the osmotic diuresis.

Solute free water reabsorption ($T^c H_2O$) was calculated from the formula:

$$T^c H_2O = \frac{U_{osm} V}{P_{osm}} - V, \text{ or } C_{osm} - V$$

where: $U_{osm}$ = urine osmolality in milliosmoles per liter.
$V$ = urine flow in milliliters per minute.
$P_{osm}$ = plasma osmolality in milliosmoles per liter.
$C_{osm}$ = osmolal clearance in milliliters per minute.

The method utilized to calculate the final value for the maximal rate of solute free water reabsorption ($T_m^c H_2O$) is that of Boyarsky and Smith,[7] in which $T_m^c H_2O$ is derived as the mean of all values of $T^c H_2O$ which fall within the rectilinear portion of the line of regression obtained by plotting $C_{osm}$ against V, irrespective of the slope of this line. This method is illustrated in Fig. 1, with data from one of our normal subjects (G. G.). $T^c H_2O$ values are represented by the degree of horizontal displacement from the isotonic line, a line established when $C_{osm}$ equals V. It will be noted that above a urine flow of approximately 8 ml. per millimeter, $T^c H_2O$ becomes relatively constant and generates a rectilinear line. The $T_m^c H_2O$ value is the mean of these various $T^c H_2O$ values (5.6, 5.5, 5.7, 5.5, 5.3, and 5.7) which compose this line and, in this subject, is 5.6 ml. per minute per 1.73 sq. M.

Plasma and urine osmolality were measured by the freezing point depression method by use of a Fiske osmometer. Hemoglobin electrophoresis was performed by the paper technique of Smith and Conley.[8] Inulin was determined by the method of Schreiner,[9] and paraaminohippurate by the method of Smith and associates,[10] except for the use of barium hydroxide and zinc sulphate in the preparation of the filtrates. The surface area was obtained by use of the nomogram devised by Hannon from the formula of DuBois.[11]

The data have been statistically evaluated by use of Fisher's t test.[12]

TABLE I. SUMMARY OF DATA OBTAINED WHEN MAXIMAL URINARY OSMOTIC CONCENTRATION AND $T_m^c H_2O$ IN 16 CHILDREN WITH SICKLE CELL ANEMIA WAS MEASURED*

| | | | | DURING HYDROPENIA | | | | | | DURING OSMOTIC DIURESIS | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 SUBJECT | 2 SEX | 3 AGE (YEARS) | 4 SURFACE AREA (SQ. M.) | 5 MAXIMUM $U_{osm}$ | 6 MAXIMUM $U_{osm}/P_{osm}$ | 7 $C_{osm}$ (ML./MIN.) | 8 V (ML./MIN.) | 9 $T^c H_2O$ (ML./MIN.) | 10 SOLUTE EXCRETION (MOSM./MIN.) | 11 $P_{osm}$† RANGE (MOSM./L.) | 12 URINE‡§ FLOW RANGE (ML./MIN.) | 13 $T_m^c H_2O$ (ML./MIN.) | 14 $C_{IN}$‖ (ML./MIN.) | 15 RPF‖ (ML./MIN.) | 16 F.F. (%) | 17 $\frac{T_m^c H_2O}{C_{IN}} \times 100$ |
| M. H. | F | 2 | 0.48 | 574 | 2.0 | 1.43 | 0.73 | 0.70 | 0.40 | 281-288 | 5.4-13.7 | 3.8 | 99.7 | 534.9 | 18.6 | 3.8 |
| L. P. | F | 2 | 0.46 | 664 | 2.4 | 2.39 | 1.01 | 1.38 | 0.66 | 278-295 | 7.8-17.4 | 5.5 | — | — | — | — |
| M. H. | M | 3 | 0.65 | 469 | 1.6 | 0.74 | 0.46 | 0.28 | 0.21 | 285-298 | 8.6-23.4 | 3.9 | — | — | — | — |
| S. I. | M | 4 | 0.65 | 525 | 1.9 | — | — | — | — | 277-300 | 7.0-11.5 | 3.4 | 80.2 | — | — | 4.2 |
| W. B. | F | 5 | 0.73 | 464 | 1.6 | 1.10 | 0.69 | 0.41 | 0.31 | 282-303 | 10.8-21.6 | 4.2 | 73.3 | 513.1 | 14.2 | 5.7 |
| T. S. | M | 5 | 0.78 | 523 | 1.9 | 1.59 | 0.85 | 0.74 | 0.44 | 279-297 | 6.6-20.5 | 3.2 | 90.2 | 495.0 | 18.2 | 3.5 |
| M. K. | F | 5 | 0.67 | 602 | 2.1 | — | — | — | — | 281-301 | 7.5-19.5 | 5.5 | — | — | — | — |
| J. W. | F | 5 | 0.71 | 596 | 2.1 | 1.00 | 0.48 | 0.52 | 0.23 | 280-303 | 14.4-21.1 | 5.2 | 106.1 | 586.3 | 18.0 | 4.9 |
| L. P. | F | 5 | 0.75 | 549 | 2.0 | — | — | — | — | 279-290 | 5.8-14.7 | 4.3 | 197.8 | — | — | 2.1 |
| B. J. | M | 6 | 0.72 | 604 | 2.1 | 1.70 | 0.79 | 0.91 | 0.47 | 280-290 | 7.2-17.0 | 6.2 | 113.7 | 626.4 | 18.1 | 5.4 |
| R. K. | F | 6 | 0.67 | 468 | 1.6 | 1.65 | 1.00 | 0.65 | 0.46 | 284-305 | 10.0-18.3 | 5.4 | 151.7 | 805.5 | 18.8 | 3.5 |
| L. P. | F | 7 | 0.78 | 429 | 1.6 | 1.11 | 0.70 | 0.41 | 0.30 | 268-300 | 8.8-30.3 | 3.5 | 141.4 | 1281.2 | 11.0 | 2.4 |
| R. M. | M | 7 | 0.72 | 644 | 2.3 | 1.27 | 0.55 | 0.72 | 0.35 | 279-295 | 10.1-28.6 | 5.3 | 228.4 | — | 13.4 | 2.3 |
| E. B. | F | 8 | 0.88 | 488 | 1.7 | 2.90 | 1.69 | 1.21 | 0.81 | 280-301 | 8.9-24.5 | 4.3 | — | 1696.0 | — | — |
| W. W. | M | 9 | 1.05 | 520 | 1.9 | 2.09 | 1.10 | 0.99 | 0.57 | 275-286 | 5.4-19.4 | 4.2 | 96.3 | 838.8 | 11.4 | 4.3 |
| D. F. | F | 13 | 1.57 | 564 | 2.0 | 1.88 | 0.95 | 0.93 | 0.53 | 280-290 | 5.9-26.4 | 3.5 | 97.2 | 630.9 | 15.4 | 3.6 |

*Data in Columns 7-10, and 12-15 corrected to 1.73 sq. M. surface area.

†Represents the hydropenic plasma osmolality and the highest plasma osmolality attained during osmotic diuresis.

‡Corresponds to the range of urine flows from which the coincident $T^c H_2O$ values were used to determine $T_m^c H_2O$.

§The figure to the left does not represent in all patients the minimum urine flow at which the value could be used to calculate $T_m^c H_2O$, for in some subjects the first urine collection was made 20 minutes after starting the mannitol infusion.

‖Measured during osmotic diuresis.

TABLE II. SUMMARY OF DATA OBTAINED WHEN MAXIMAL URINARY OSMOTIC CONCENTRATION AND $T^c_m H_2O$ IN 12 NORMAL CHILDREN WAS MEASURED*

| | | | | DURING HYDROPENIA | | | | | | DURING OSMOTIC DIURESIS | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 SUBJECT | 2 SEX | 3 AGE | 4 SURFACE AREA (SQ. M.) | 5 MAXIMUM $U_{osm}$ | 6 MAXIMUM $U_{osm}/P_{osm}$ | 7 $C_{osm}$ (ML./MIN.) | 8 V (ML./MIN.) | 9 $T^c H_2O$ (ML./MIN.) | 10 SOLUTE EXCRETION (MOSM./MIN.) | 11 $P_{osm}$† RANGE (MOSM./L.) | 12 URINE‡§ FLOW RANGE (ML./MIN.) | 13 $T^c_m H_2O$ (ML./MIN.) | 14 $C_{IN}$‖ (ML./MIN.) | 15 RPF‖ (ML./MIN.) | 16 F.F. (%) | 17 $\frac{T^c_m H_2O}{C_{IN}} \times 100$ |
| R. R. | M | 14 mo. | 0.41 | 880 | 3.2 | — | — | — | — | 273-305 | 8.4-23.5 | 5.1 | 94.5 | — | — | 5.4 |
| D. S. | F | 3 yrs. | 0.62 | 964 | 3.5 | 1.76 | 0.50 | 1.26 | 0.48 | 275-299 | 17.1-27.1 | 5.2 | 93.5 | — | — | 5.5 |
| T. P. | F | 3 yrs. | 0.62 | 1,060 | 3.9 | 2.10 | 0.53 | 1.57 | 0.59 | 272-290 | 5.3-15.6 | 5.6 | 75.4 | 573.5 | 13.2 | 7.4 |
| A. G. | M | 6 yrs. | 0.73 | 1,125 | 4.1 | 3.02 | 0.74 | 2.28 | 0.83 | 275-292 | 9.1-24.0 | 7.5 | 144.9 | — | — | 5.2 |
| S. L. | M | 7 yrs. | 0.95 | 1,007 | 3.6 | 2.77 | 0.79 | 1.98 | 0.75 | 276-286 | 7.9-15.6 | 7.5 | — | — | — | — |
| P. N. | F | 7 yrs. | 0.90 | 1,140 | 4.0 | — | — | — | — | 282-297 | 8.4-24.7 | 4.2 | — | — | — | — |
| J. R. | M | 9 yrs. | 1.23 | 1,144 | 4.1 | 2.35 | 0.60 | 1.75 | 0.64 | 274-295 | 4.2-28.7 | 6.9 | 93.5 | 461.1 | 20.1 | 7.4 |
| S. G. | F | 10 yrs. | 1.42 | 807 | 2.8 | 2.72 | 0.96 | 1.76 | 0.75 | 277-292 | 8.1-22.6 | 4.2 | 105.7 | 416.8 | 25.3 | 4.0 |
| E. M. | M | 10 yrs. | 1.13 | 1,099 | 4.0 | 3.16 | 0.79 | 2.37 | 0.86 | 276-289 | 4.1-20.3 | 4.0 | 107.3 | 703.8 | 15.2 | 3.7 |
| N. S. | M | 12 yrs. | 1.14 | 1,038 | 3.7 | 1.54 | 0.42 | 1.12 | 0.43 | 281-296 | 3.1-18.7 | 3.6 | — | — | — | — |
| L. G. | F | 11 yrs. | 1.2 | 1,210 | 4.2 | 1.55 | 0.42 | 1.13 | 0.44 | 284-297 | 5.7-20.7 | 3.4 | 88.5 | 528.2 | 16.7 | 4.0 |
| G. G. | F | 15 yrs. | 1.57 | 1,207 | 4.4 | 2.42 | 0.58 | 1.84 | 0.66 | 275-290 | 8.5-23.7 | 5.6 | — | — | — | — |

*Data in Columns 7-10, and 12-15 corrected to 1.73 sq. M. surface area.

†Represents the hydropenic plasma osmolality and the highest plasma osmolality attained during osmotic diuresis.

‡Corresponds to the range of urine flows from which the coincident $T^c_e H_2O$ values were used to determine $T^c_m H_2O$.

§The figure to the left does not represent in all patients the minimum urine flow at which the value could be used to calculate $T^c_m H_2O$, for in some subjects the first urine collection was made 20 minutes after starting the mannitol infusion.

‖Measured during osmotic diuresis.

TABLE III. COMPARISON OF THE PERTINENT DATA OBTAINED IN CHILDREN WITH SICKLE CELL ANEMIA WITH THE DATA OBTAINED IN NORMAL CHILDREN*

| | DURING HYDROPENIA | | | | | DURING OSMOTIC DIURESIS | | | |
|---|---|---|---|---|---|---|---|---|---|
| GROUP | 1 MAXIMAL $U_{osm}$ (MOSM./L.) | 2 $C_{osm}$ (ML./MIN.) | 3 V (ML./MIN.) | 4 $T^c H_2O$ (ML./MIN.) | 5 SOLUTE EXCRETION (MOSM./MIN.) | 6 $T^c_m H_2O$ (ML./MIN.) | 7 $C_{IN}$ (ML./MIN.) | 8 $\frac{T^c_m H_2O}{C_{IN}} \times 100$ | 9 INC. IN PLASMA OSMOLALITY (MOSM./L.) |
| *Normal.*— | | | | | | | | | |
| Number | 12 | 10 | 10 | 10 | 10 | 12 | 8 | 8 | 12 |
| Range | 807-1,210 | 1.55-3.16 | 0.42-0.96 | 1.12-2.37 | 0.43-0.86 | 3.4-7.5 | 75.4-144.9 | 3.7-7.4 | 10-32 |
| Mean | 1,056.7 | 2.33 | 0.63 | 1.70 | 0.64 | 5.2 | 100.4 | 5.3 | 16.2 |
| S.D. | ±128 | | ±0.19 | ±0.43 | ±0.17 | ±1.4 | ±20.5 | ±1.45 | ±5.8 |
| *Sickle Cell Anemia.*— | | | | | | | | | |
| Number | 16 | 13 | 13 | 13 | 13 | 16 | 12 | 12 | 16 |
| Range | 429-664 | 0.74-2.90 | 0.46-1.69 | 0.28-1.38 | 0.21-0.81 | 3.2-6.2 | 73.3-228.4 | 2.1-5.7 | 19.1 |
| Mean | 542.6 | 1.60 | 0.84 | 0.75 | 0.44 | 4.5 | 122.9 | 3.5 | 19.1 |
| S.D. | ±68.7 | | ±0.32 | ±0.32 | ±0.17 | ±0.9 | ±15.2 | ±1.54 | ±4.2 |
| p value | <0.001 | 0.6 | 1.8 | <0.001 | 0.01 | 0.1 | 0.2 | 0.4 | 0.7 |

*All data except Columns 1 and 9 corrected to 1.73 sq. M. surface area.

## RESULTS AND COMMENTS

A summary of the data obtained from measurement of maximal urinary osmotic concentration and $T^c_m H_2O$ in children with sickle cell anemia and normal children is recorded in Tables I and II, respectively. A statistical analysis of the pertinent data is presented in Table III.

In Fig. 2 the maximal urinary osmotic concentration is depicted in milliosmoles per liter attained in the hydropenic state. The range for the normal children was from 807 mOsm. per liter to 1,210 mOsm. per liter, with a mean of 1,056 ± 128 (S.D.) mOsm. per liter, in contrast to a range of 420 to 664 mOsm per liter and a mean of 542 ± 68 (S.D.) mOsm. per liter in the 16 children with sickle cell anemia. An impairment in concentrating ability in children with sickle cell anemia is revealed by this test. This defect is graphically evident (Fig. 2) and statistically significant (p value 0.001). The degree of defect in concentrating ability in our 16 subjects is similar to that reported by others.[1-4]

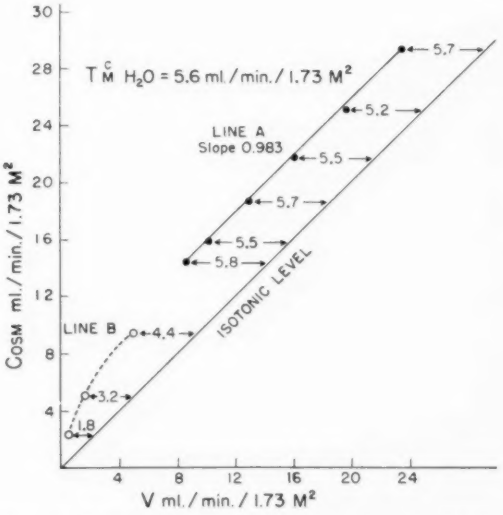


Fig. 1.—$T^c H_2O$ values in Subject G. G. (15-year-old normal child), indicating method used to determine $T^c_m H_2O$

Confirmation of the presence of a concentrating defect during hydropenia is obtained when the actual amount of solute free water being reabsorbed ($T^c H_2O$) during antidiuresis in the 2 groups is compared (Tables I and II, Column 9), when, presumably the need to conserve water is identical. The average value for the normal children is 1.7 ml. per minute per 1.73 sq. M., whereas in the children with sickle cell anemia the average rate of solute free water reabsorption during antidiuresis is only 0.7 ml. per minute per 1.73 sq. M., less than 50 per cent of normal (Table III, Column 4). The p value is again highly significant (<0.001).

Our data substantiate the current view[1-4] that an impairment in renal concentrating ability in the antidiuretic state is a universal finding in subjects with sickle cell anemia and that the onset of this defect is in early childhood.

Since presumably the same mechanisms which are responsible for water reabsorption during hydropenia operate in water reabsorption during osmotic diuresis, it would be anticipated that, in disease states in which maximum urinary osmotic concentration is less than normal, there would be a similar alteration in $T^c_m$ $H_2O$. The relationship of these 2 functions in disease states has not been adequately studied, but an examination of the data, reported by

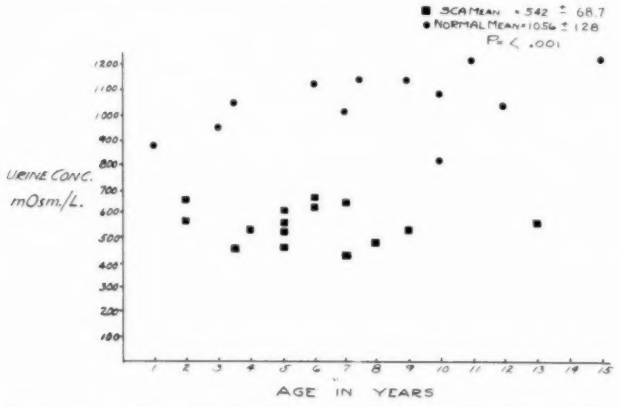


Fig. 2.—Comparison of maximal urinary osmotic concentration (hydropenia) in normal children and in children with sickle cell anemia.

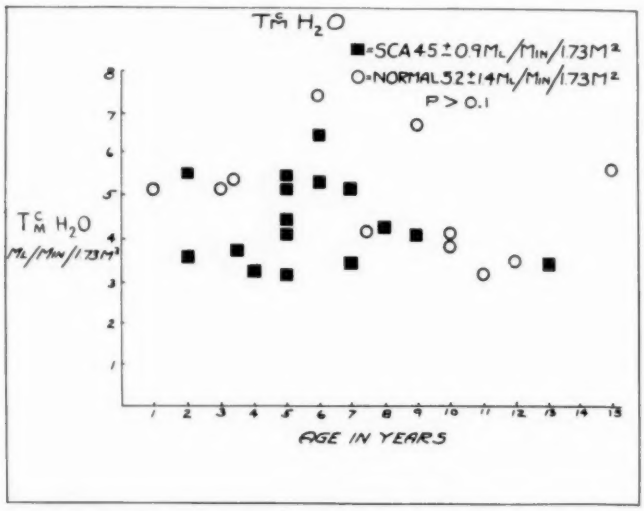


Fig. 3.—Comparison of $T^c_m$ $H_2O$ in normal children and in children with sickle cell anemia.

Baldwin and co-workers,[13] in which maximum osmotic U:P ratio and $T^c_m$ $H_2O$ were determined in adults with acute and chronic glomerulonephritis and acute renal failure reveals that a parallelism in impairment was invariably present.

When we examine the $T^c_m$ $H_2O$ values in children with sickle cell anemia (Fig. 3), we find that the range, 3.2 to 6.2 ml. per minute per 1.73 sq. M., is similar to the range, 3.4 to 7.5 ml. per minute per 1.73 sq. M., found in normal

children. The mean in the group with sickle cell anemia is 4.5 ml. per minute per 1.73 sq. M. and, thus, slightly lower than the mean of 5.2 ml. per minute per 1.73 sq. M. in the normal group. However, the p value is greater than 0.1; therefore, the difference in $T^c_m H_2O$ between the normal children and the children with sickle cell anemia is not statistically significant.

Our data reveal that at low urine flows (hydropenic state) in children with sickle cell anemia there is an impairment in water reabsorptive ability, whereas at high urine flows (osmotic diuresis) water reabsorption occurs at normal rates. An appraisal of our data relative to differences in osmolal clearance, solute excretion, and glomerular filtration rate (Table III) between the normal children and the children with sickle cell anemia does not uncover an explanation for a normal $T^c_m H_2O$ in the group with sickle cell anemia. A consideration of a possible explanation for this apparent inconsistency in renal concentrating ability, i.e., normal $T^c_m H_2O$ and subnormal maximal urinary osmotic concentration, will be developed (see below). Before this is done, several aspects of our results deserve further comment.

As originally observed by Zak and co-workers[14] and substantiated by other investigators,[7, 13] there is a lack of constancy of $T^c H_2O$ in a significant number of individuals when $T^c H_2O$ is measured over a wide range of urine flow. $T^c H_2O$ values increase or decrease progressively as urine flow increases. If $T^c H_2O$ were a constant value during high levels of osmotic diuresis, the slope of the line of regression generated by $T^c H_2O$ (Fig. 1) would be 1.000, or within a range of 0.950 to 1.050, with allowance for a reasonable variation of 5 per cent for methodology.[14] In Table IV we have listed the statistical values of slopes of our subjects as well as those from the adult studies of Boyarsky and Smith[7] and Zak, Brun, and Smith.[14] It will be noted that in each group a significant number of slopes exceed the limits of a 5 per cent range of variance. Why this variation in $T^c H_2O$ occurs in some subjects is unknown, but, because it occurs, it raises a question as to how valid a comparison can be made between $T^c_m H_2O$ values in 2 individuals, if a difference in slope exists. This aspect is being explored because a difference in slopes between the normal children and those with sickle cell anemia might conceal actual differences in $T^c_m H_2O$. However, the range, mean, and standard deviation of the slopes for the group with sickle cell anemia are so similar to the values for normal children and normal adults that variations in slope will not provide an explanation for normal $T^c H_2O$ in children with sickle cell anemia (Table IV).

$T^c_m H_2O$ capacity in children, when corrected for surface area, is an early maturing function with a mean of 5.2 ± 1.4 (S.D.) ml. per minute per 1.73 sq. M. This compares favorably with the mean of 5.1 ± 1.5 ml. per minute per 1.73 sq. M. reported by Zak, Brun, and Smith[14] in 21 normal adults and the mean of 4.99 ± 0.85 ml. per minute per 1.73 sq. M. reported by Boyarsky and Smith[7] in 9 normal adults.

We have, as have Zak, Brun, and Smith,[14] calculated the ratio of $T^c_m H_2O$ to $C_{H_2O}$ (Tables I and II, Column 17). However, we feel that physiologic implications derived from a consideration of this ratio would only be justified if

it were established that from each 100 ml. of glomerular filtrate the maximal amount of solute free water ($T^c_m$ $H_2O$) was being constantly reabsorbed. Present evidence suggests that this is not the case.[15] Indeed, when we actually measure the amount of solute free water reabsorption below osmotic diuretic levels of urine flow (e.g., during hydropenia, Tables I and II, Column 9), the rate of reabsorption is far below $T^c_m$ $H_2O$. That physiologic interpretations based on this ratio are of questionable validity is illustrated by the data on Subject R. M. (Table I). His $T^c_m$ $H_2O$ to $C_{IN}$ ratio is 2.3, which is markedly reduced. However, his absolute $T^c_m$ $H_2O$, 5.3, is well within the normal range. The low ratio is the result of an elevated glomerular filtration rate (not an uncommon finding in children with sickle cell anemia[16]), and there is no apparent basis for the conclusion that his functional tubular damage is more severe than that in those individuals with a normal $T^c_m$ $H_2O$ to $C_{IN}$ ratio.

TABLE IV. COMPARISON OF THE SLOPE OF THE LINE FROM WHICH $T^c_m$ $H_2O$ WAS CALCULATED IN NORMAL ADULTS, NORMAL CHILDREN, AND CHILDREN WITH SICKLE CELL ANEMIA

| SUBJECTS | NO. | RANGE WITHIN 5% (0.950-1.050) | RANGE | MEAN AND S.D. |
|---|---|---|---|---|
| Normal adults (Zak, Brun, and Smith[14]) | 21 | 9 | 0.863-1.168 | 1.033 ± 0.075 |
| Normal adults (Boyarsky and Smith[7]) | 12 | 3 | 0.894-1.269 | 1.114 ± 0.115 |
| Normal children | 12 | 8 | 0.918-1.232 | 1.082 ± 0.082 |
| Children with sickle cell anemia | 16 | 7 | 0.964-1.268 | 1.012 ± 0.086 |

The increase in plasma osmolality from the hydropenic state to the height of osmotic diuresis in the group with sickle cell anemia averaged approximately 19 mOsm. per liter (Table III, Column 9). In spite of this marked increase in plasma osmolality, in none of these children were symptoms suggestive of a clinical crisis of sickle cell anemia produced. Although the duration of the elevated plasma osmolality was short, we feel that the magnitude of the increase was sufficient to make it unlikely that a rise in plasma osmolality is a factor in the occurrence of vascular occlusive phenomenon in sickle cell anemia. That hyperosmolality plays a role in sickle cell crises is the assumption upon which hydration therapy (oral or intravenous), a form of therapy of questionable value, has been based.

In those few individuals with sickle cell hemoglobin-C disease and sickle cell thalassemia in whom concentration tests in the hydropenic state have been performed, an impairment has been noted.[3, 6] In 3 children with sickle cell–hemoglobin-C disease we obtained maximal urinary osmotic concentrations and $T^c_m$ $H_2O$ values as follows: a 7-year-old girl, 618 mOsm. per liter and 6.3 ml. per minute per 1.73 sq. M.; an 8-year-old boy, 656 mOsm. per liter and 5.7 ml. per minute per 1.73 sq. M.; and an 11-year-old boy, 531 mOsm. per liter and 3.9 ml. per minute per 1.73 sq. M. In a 2½-year-old child with probable sickle cell–thalassemia (S-pattern on electrophoresis, hemoglobin 10 Gm., no sickling in peripheral smear, but some hypochromasia and ovalocytosis –

mother, sickle cell trait; father, not available) values of 758 mOsm. per liter and 6.3 ml. per minute per 1.73 sq. M. were obtained. Thus, again, we have the finding of a normal $T_m^c H_2O$ and a diminished maximum urinary osmotic concentration associated with a sickle cell disease state. (Table V.)

## DISCUSSION

Our data suggest an incongruity in renal concentrating power in children with sickle cell anemia, in that their maximal urinary osmotic concentration is reduced while the maximal rate of solute free water reabsorption achieved during osmotic diuresis, $T_m^c H_2O$, is essentially normal. In order to develop a completely valid explanation for this apparent dichotomy we would need to know in detail: how renal concentration is accomplished in normal individuals, how the pathophysiology of sickle cell anemia produces

TABLE V. MAXIMUM URINARY OSMOTIC CONCENTRATION AND $T_m^c H_2O$ IN THREE CHILDREN WITH SICKLE CELL–HEMOGLOBIN-C DISEASE AND ONE CHILD WITH SICKLE CELL–THALASSEMIA

| 1 SUBJECT | 2 SEX | 3 AGE (YEARS) | 4 DIAGNOSIS | 5 MAXIMUM $U_{osm}$ (MOSM./L.) | 6 MAXIMUM $U_{osm}/P_{osm}$ | 7 $T_m^c H_2O$ (ML./MIN./ 1.73 SQ. M.) |
|---|---|---|---|---|---|---|
| A. D. | F | 7 | Sickle cell–Hb. C | 618 | 2.2 | 6.3 |
| W. P. | M | 8 | Sickle cell–Hb. C | 656 | 2.3 | 5.7 |
| P. B. | M | 11 | Sickle cell–Hb. C | 531 | 1.8 | 3.9 |
| W. B. | M | 2½ | Sickle cell–thalassemia | 758 | 2.6 | 6.3 |

an impairment of the ability to attain a normal maximal urinary osmotic concentration, and whether the factors which govern maximal urinary osmotic concentration are identical with those controlling $T_m^c H_2O$. Our present state of knowledge does not permit absolute answers to these fundamental questions, so a definitive physiologic interpretation of our results cannot be presented. However, an analysis of the question raised by our observations is in order and in the following discussion we will examine current hypotheses relative to normal renal concentrating mechanisms and the pathogenesis of sickle cell anemia as they pertain to our observations in an effort to define the problem and to lay the foundation for a speculative interpretation of our observations.

*Pathogenesis of Sickle Cell Anemia.*—According to current concepts, summarized by Smith and Conley,[17] the pathologic manifestations of sickle cell anemia are the result of the presence of sickled erythrocytes in the circulation and are mediated through 2 features of the intravascularly sickled erythrocyte. First, sickled erythrocytes are mechanically more fragile than normal erythrocytes and have a decreased life span in vivo of sufficient degree to prevent maintenance of a normal hemoglobin level.[18-22] Thus, individuals with sickle cell anemia are subject to all the pathologic features of a chronic hemolytic anemia. Second, there is a tendency for blood vessels to become plugged with sickled cells, with the accompanying erythrostasis and/or thrombosis which results in local ischemia or infarction.[18, 23] The net effect of this so-called vascular

occlusive phenomenon is that the tissues supplied by the involved vessels sustain anoxic damage. All of the other factors thus far delineated as playing a role in the pathologic physiology of sickle cell anemia, i.e., infection, pH changes, tissue oxygen tension levels, etc.,[24, 25] are secondary ones which operate through augmenting either the degree of intravascular sickling, the rate of destruction of the sickled cells, or the tendency for the occurrence of vascular occlusion.

Anemia can be immediately dismissed as a causative factor in hyposthenuria, in that a similar impairment of renal concentrating ability has not been discovered in other chronic anemias[3] and reduced maximal urinary osmotic concentration has been described in individuals with sickle cell trait,[3, 4] an entity in which anemia is not a feature. Thus by exclusion, we are left with the probability that the effect of intravascular sickling on renal concentrating power measured by maximal urinary osmotic concentration is mediated through dynamic alterations in intrarenal circulation or the production of anoxic damage to renal tubular cells. How this might occur must be considered within the framework of the mechanics of normal renal concentrating activity.

*Normal Renal Concentrating Mechanism.*—Current thought relative to the mechanism, by means of which a concentrated urine is elaborated, is based upon the action of the loop of Henle as a countercurrent multiplier system, as first proposed by Hargitay and Kuhn.[26] At least 4 different opinions have been published relative to the precise details of the operation of the countercurrent loop scheme.[15, 27-29] However, in each of these concepts a combination of 4 variables at the renal level appears to play the major role in the determination of the ultimate maximal urinary osmotic concentration that is attainable. They are: (1) rate of medullary blood flow, (2) concentration and volume of fluid reaching the collecting ducts, (3) permeability of the renal tubular epithelium to water, and (4) amount of sodium reaching and transported by the loop.

Could intravascular sickling adversely affect one or more of these factors in such a manner that solute free water reabsorption would be normal during osmotic diuresis and subnormal during hydropenia? We will consider these factors individually to ascertain whether a reasonable speculation can be developed to account for our observations.

*Rate of medullary blood flow:* To date, renal medullary blood flow has not been measured and, indeed, we have no sound basis for even the estimation of its magnitude, but theoretic considerations, as outlined by Berliner,[15] suggest that the level of urine concentration attainable is inversely proportional to the square of the medullary blood flow. Just what effect intravascular sickling has upon renal medullary blood flow is an unknown quantity and complicated by the fact that total renal blood flow is increased in some children with sickle cell anemia,[16] a finding that is unexplained and inconsistent with the vascular obstruction that we anticipate in sickle cell anemia. Even if medullary blood flow is increased in children with sickle cell anemia coincident with an increase in total renal blood flow, this would not serve as a likely explanation for the concentration defect in sickle cell anemia. In adults, the concentration defect

is evident, but total renal blood flows are normal or reduced.[30] This leaves the unlikely possibility that an independent increase in medullary blood flow (independent of total renal blood flow) is a consistent feature of sickle cell anemia, unlikely because the ultimate effect of intravascular sickling in most organs is one of impaired circulation.[17]

*Concentration and volume of fluid reaching the collecting ducts:* If the fluid presented to the segment of the nephron (distal tubule and/or collecting ducts) responsible for the elaboration of a concentrated urine is less concentrated than normal or of increased volume, it is conceivable that the maximal concentration attainable could be decreased. This could be the result either of an elevated glomerular filtration rate or of a decrease in water and solute reabsorption in the proximal convoluted tubule. Glomerular filtration rate is increased in some children with sickle cell anemia,[16] but this is not a consistent finding (Table II), and it is not present in adults with sickle cell anemia who are hyposthenurie.[30] As to proximal tubular functional impairment, other indices of tubular function at this level which have been measured are normal.[4] Of greater significance is the probability that the circumstances, under which a "flooded" concentrating mechanism would be most likely to occur, would be favorable during osmotic diuresis when the urine flow is high rather than during hydropenia when the flow is minimal. However, this is not in keeping with our data, for reabsorption of water in our subjects was normal during osmotic diuresis and decreased during hydropenia (Table I, Column 9). Therefore, a consideration of the concentration and volume of fluid which reaches the collecting ducts does not provide us with the answer we are seeking.

*Permeability of the renal tubular epithelium to water:* The role of membrane permeability to water is closely related to that of the antidiuretic hormone in the concentrating kidney. The assumed function of the antidiuretic hormone is to render various segments of the renal tubular epithelium permeable to water.[15] Membrane permeability and/or the response to antidiuretic hormone could be impaired by anoxic damage secondary to the vascular occlusive effects of sickle cell anemia. Indeed, evidence that there is a decreased responsiveness to injected Pitressin has been reported. Kunz and co-workers[1] and McCrory and associates[2] found that when Pitressin was injected during a water diuresis in children with sickle cell anemia the magnitude of antidiuresis, the duration of antidiuresis, and the level of urine osmotic concentration attained were abnormal in the majority of the children studied. However, finding decreased responsiveness to Pitressin does not necessarily imply that this is a primary factor in the concentrating defect, since the findings can be interpreted as simply reflections of a concentrating defect on another basis.

If the renal tubular epithelium were less permeable to water or less responsive to antidiuretic hormone, this impaired "permeability" would be more evident at high rates of reabsorption. Again our data indicate that the reverse is true; water reabsorption is normal at high rates of urine flow and reduced when the rate of urine flow is minimal.

To achieve a normal $T^c_m$ $H_2O$ a full response to antidiuretic hormone must occur and some investigators,[13, 31] to insure this, have infused Pitressin while measuring this moiety. The finding of normal $T^c_m$ $H_2O$ in children with sickle cell anemia, without infusion of Pitressin, is further evidence that altered membrane permeability (or diminished response to antidiuretic hormone) is not a likely explanation for our observations.

*Amount of sodium reaching and transported by the loop:* Since the level of hypertonicity, established in the renal medulla by the deposition of sodium salts, determines the amount of water that will diffuse out of the collecting ducts, the amount of sodium reaching and transported by the loop is a primary factor in the determination of the maximal urinary osmotic concentration. We have no data which indicate that the amount of sodium delivered to or transported by the loop is diminished in sickle cell anemia. We suppose, however, that the renal epithelial cells of the loop sustain anoxic damage secondary to the vascular occlusive phenomenon associated with intravascular sickling, so that the renal tubular mechanism, by which sodium is transported from the ascending loop, is impaired. The results of this impairment would be failure of the medullary interstitium to attain as high a level of hypertonicity as would be attained in the normal kidney and reduction in maximal urinary osmotic concentration. This would be a feasible explanation for an impairment of maximal urinary osmotic concentration.

Can we now account for a normal $T^c_m$ $H_2O$? We know that during osmotic diuresis, induced by the intravenous infusion of mannitol, there is a decrease in tubular reabsorption of sodium with a progressively increasing rate of sodium excretion by the kidneys.[32-34] It is conceivable that, as a result of this increasing sodium excretion, less sodium is transported to the interstitium and the degree of hypertonicity in the renal medulla is normally less during osmotic diuresis than during antidiuresis. (Crawford, Parisi, Meara, and Terry[35] have reported that this is true in rabbits.) If this is so, the amount of sodium transported and the level of hypertonicity attained normally during osmotic diuresis might be well within the capacity of the damaged tubular cells of individuals with sickle cell anemia, thus $T^c_m$ $H_2O$ would be within normal limits. Considerable additional information must be available relative to both normal humans and individuals with sickle cell anemia before this speculation can be accepted; however, at the moment it appears to be an explanation that is in agreement with current concepts.

One final factor relative to an explanation for our data deserves consideration. In order to compare $T^c_m$ $H_2O$ in the 2 groups of children, we have followed the conventional practice of correcting the observed data to 1.73 sq. M of body surface area. Recently the validity of relating physiologic functions to surface area has been challenged by Oliver, Graham, and Wilson.[36] The objections raised by these authors were: (1) it has not been adequately demonstrated that the rates of various physiologic functions are proportional to surface area, and (2) the values commonly used for surface area are not accurately measured but are estimated from normograms. In a more recent

editorial, Forbes[37] points out that values corrected by means of raising a variable to the nth power (as is done in the method we used for surface area estimation) tend to decrease the variability of the observations thus corrected. Finally, according to the earlier data of Boyd,[38] younger children introduce a larger correction error than do older children.

The question arises as to whether the utilization of surface area as a reference standard has artificially raised the $T_m^c$ $H_2O$ values for the group with sickle cell anemia into the normal range.

In comparisons of renal clearance in individuals of different sizes, the need for some reference standard is clearly indicated. It has been a widely accepted and utilized practice to correct renal clearance data on the basis of surface area.

In an effort to arrive at the best reference standard for comparisons of renal function studies in children with those in adults, Rubin, Bruck, and Rapoport[39] related glomerular filtration rates to body height, body weight, kidney weight, and surface area. These investigators found that the best correlation with adult values was obtained when kidney weights were used as the reference standard, in spite of the fact that the kidney weights used were not measured but obtained from data in the literature. When body weight was utilized the values were considerably above the adult range in children up to 3 years of age. When surface was the reference unit the values for glomerular filtration were low in children in early infancy and reached the adult range by the end of the second year of life.

The finding of renal clearance values consistently in the adult range in children above the age of 2 years when surface area is used is of significance in consideration of our data. Children with sickle cell anemia commonly show a retardation in growth, hence, when a child with sickle cell anemia is compared with a normal child of the same age, a larger correction factor would be employed. However, since above the age of 2 years other measured renal clearances reach an adult level when corrected for surface area it appears that age per se is not a significant factor in the age range used in our study. The fact that the children with sickle cell anemia have smaller surface area and, hence, larger correction factors is not likely to affect spuriously their $T_m^c$ $H_2O$ values any more than it affects the values of the normal children who were of varying sizes.

Homer Smith[40] has concluded, from an appraisal of the various bases upon which renal functions in individuals might be compared, that "the surface area basis of comparison cannot be improved upon at the present time." Although to suggest that an unreliable standard is better than none is not warranted, having presented the limitations and objections involved, we feel justified in reporting and interpreting our data on the basis of a standard, i.e., surface area, which has yielded a wealth of reasonable and acceptable data relative to renal function in children. We must conclude that the use of surface area as a corrective factor does not account for the normal $T_m^c$ $H_2O$ values in children with sickle cell anemia.

## SUMMARY AND CONCLUSIONS

1. Solute free water reabsorption during osmotic diuresis ($T^c_m$ $H_2O$) was found to be substantially normal in 16 children with sickle cell anemia, in spite of a definite reduction in maximal urinary osmotic concentration.

2. A similar picture was present in 3 children with sickle cell–hemoglobin-C disease and in 1 child with sickle cell–thalassemia.

3. Speculation as to a possible explanation for this dissociation of renal water reabsorptive ability as indicated by these 2 indices has been presented.

4. $T^c_m$ $H_2O$ values in 12 normal children are in agreement with those reported in normal adults.

5. It is improbable that an increase in plasma osmolarity plays a role in the vascular-occlusive phenomenon in sickle cell disease states.

We wish to express our gratitude to Dr. Yoshikazu Morita for advice and for the use of laboratory space and equipment, and to Mrs. Maxine Adams and Mrs. Juanita Maxwell for technical assistance.

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# LABELING OF RED BLOOD CELLS WITH RADIOACTIVE DIISOPROPYLFLUOROPHOSPHATE ($DFP^{32}$): EVIDENCE FOR AN INITIAL RELEASE OF LABEL

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RADIOACTIVE diisopropylfluorophosphate ($DFP^{32}$) has been frequently used as a label for blood cells and proteins. It is a basic assumption that $DFP^{32}$ is irreversibly bound to a protein site, no label being released until the protein breaks down. Contrary to this assumption, we observed that there is a definite and, sometimes, a marked release of the label from red cells during a short period after the labeling. Bove and Ebaugh[1] and van Putten[2] have recently reported similar findings.

The practical use of $DFP^{32}$ as a red cell tag depends, to a considerable degree, on its behavior during the first few days after the labeling, and also on its suitability as an in vitro tag. Therefore, these two questions were investigated in the present study.

## MATERIALS AND METHODS

In vivo experiments in man were carried out in male medical students without anemia. For animal experiments we used Sprague-Dawley rats weighing 300 to 400 grams, and either sex of white New Zealand rabbits weighing about 2,000 grams.

*Nonradioactive DFP* was synthesized by Dr. Fischer in the Department of Biochemistry, University of Washington. It was assayed by a modification of the method of Marsh and Neale[3] suggested to us by Dr. Dixon in the same department.† The result was checked against the total phosphorus content in the preparation, and the DFP by these measurements proved to be at least 95 per cent pure. It was dissolved in water-free propylene glycol to a concentration of 400 μg per ml.

*Radioactive $DFP^{32}$*‡ was diluted in water-free propylene glycol. The concentration of $DFP^{32}$ and the total phosphorus content were determined in the same manner as were those of the nonradioactive DFP. The radioactivity was determined by comparison with standards of similar energy. These measurements were made 2 to 3 days after the synthesis. The purity in terms of phosphorus was found to be 70 per cent or better, and the specific activity was 150 to 200 μc per mg. The ampules were stored in the cold, and the $DFP^{32}$ was used

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†A description of this technique can be obtained from us.

‡Radioactive $DFP^{32}$ was supplied by Manning Research Laboratory, 27 Baldwin Road, Waltham 54, Mass.

immediately after the ampules had been opened. The experiment in Fig. 4 was made with one batch of $DFP^{32}$ from The Netherlands.* Eleven days after synthesis the purity in terms of phosphorus was 70 per cent, and the specific activity was 130 $\mu$c per mg.

*Radioactive Diisopropylphosphate ($DIP^{32}$).*—$DFP^{32}$ was hydrolyzed in 1:10 v/v of 1N NaOH for 30 minutes at room temperature. No $DFP^{32}$ could then be detected, and the solution was adjusted to physiological pH and ionic strength before injection.

*Radioactive chromium* ($Na_2Cr^{51}O_4$)† had a specific activity of 30 to 40 $\mu$c per microgram Cr. It was dissolved in saline.

*Labeling in vivo* was done by intramuscular injection in rats and by intravenous injections in humans.

*Labeling In Vitro.*—Citrated blood (containing 1:10 v/v of 3.13 per cent sodium citrate dihydrate) or ACD blood was centrifuged at 1,500 r.p.m. (500 g) for 10 minutes at 4° C. The plasma was discarded, the red cells were washed once in a volume of saline equal to the plasma volume, and resuspended in saline to a hematocrit of about 60. After incubation with $DFP^{32}$ in a stoppered vessel, the cells were washed twice in saline (unless otherwise stated). In some experiments $Cr^{51}$ in a concentration of less than 0.10 $\mu$g per milliliter red cells was added before incubation. For transfusion, the red cells were resuspended in saline to the original volume of blood.

*Collection of Blood Samples After Labeling.*—Human blood was drawn by venipuncture into dried oxalate mixture, 2 mg. per milliliter blood.[4] Rabbit blood was collected from ear veins or by heart puncture; the same anticoagulant was used. Rat blood was collected from vena cava; 1:20 v/v of 5 per cent sodium versenate was used as anticoagulant.[5]

*Processing of Blood Samples.*—One milliliter whole blood was mixed with 9 ml. 0.9 per cent saline and centrifuged at 1,200 r.p.m. (340 g) for 5 minutes. The supernatant was discarded, the washing was repeated twice, and the red cells were suspended in saline to 2 to 10 ml. volume, depending on their radioactivity.

Aliquots of red cells were also precipitated with trichloroacetic acid (TCA). The washed red cells from 1 ml. blood were suspended in 1 ml. saline, and 2 ml. 20 per cent TCA was added. Fifteen minutes later, the precipitate was sedimented by centrifugation for 3 minutes at 1,000 r.p.m. (235 g), washed 2 times in 5 ml. portions of 10 per cent TCA, dissolved in 2 ml. 2N NaOH, and made up to 3 to 10 ml. volume with saline.

*Hematocrit* was measured in a Wintrobe tube[4] after centrifugation at 3,000 r.p.m. (2,110 g) for 30 minutes.

*Measurement of the Radioactivity.*—One to 2 ml. of the sample was pipetted into counting planchets, 0.2 to 0.5 ml. 0.1N NaOH was added,[6] and, a few minutes later, 1 ml. nonradioactive blood was added. The internal diameter of the planchets was 24 mm., and the dry weight of the samples was 300 to 600 mg. The samples were dried at room temperature and counted in an end window Geiger Muller counter. A total of 4,096 counts were compiled for each sample. The background radioactivity was 24 to 26 and 14 to 16 c.p.m. in the 2 counters used. The samples gave at least 50 net counts per minute. In some experiments the activity was lower, and 10 ml. liquid samples were then counted in a Veall tube.[7] The background radioactivity was here 9 to 14 c.p.m., and at least 2,000 counts were compiled for each sample. The $Cr^{51}$-labeled samples were pipetted into counting vials, 1 to 2 ml. in each, and counted in a scintillation well counter. The background radioactivity was 73 to 90 and 54 to 58 c.p.m. in the 2 counters that were used. At least 4,000 counts were compiled for each sample. Correction for physical decay was made on the basis of the decay of standards of $DFP^{32}$ and $Cr^{51}$ which were counted with the samples. In some experiments it was necessary to correct for cross-counting between the 2 isotopes: the $Cr^{51}$ count had to be reduced with 6 per cent of the $DFP^{32}$ count, and the $DFP^{32}$ count with 0.1 per cent of the $Cr^{51}$ count. When the activity was referred to red cells, the results were corrected for variations in the hematocrit.

*Supplied by Dr. J. A. Cohen, Medical Biological Laboratory, National Defense Council, Rijswijk, The Netherlands.

†Radioactive chromium was supplied by Abbott Laboratories, Oak Ridge, Tenn.

## RESULTS

*$DFP^{32}$ Binding Capacity of Red Cells In Vitro.*—DFP combines only with certain proteins,[6, 8] and red cells should, therefore, have a limited capacity for carrying $DFP^{32}$ in an irreversible bond. To evaluate this capacity, aliquots of 1 ml. human red cells were incubated for 15 hours at 4° C. with varying concentrations of $DFP^{32}$. The red cells were then washed 3 times in 10 ml. saline, and aliquots were treated with TCA to eliminate nonprotein-bound $DFP^{32}$.

Fig. 1 shows the result of this experiment. There appears to be a relative saturation point at 0.14 μg $DFP^{32}$ per milliliter packed red cells, at which point about 90 per cent of available $DFP^{32}$ was bound. When the amount of $DFP^{32}$ was increased above this point, the relative uptake decreased, and part of the total uptake was no longer bound to proteins. The protein-bound $DFP^{32}$ continued to increase, and in a straight line fashion with increasing amounts of $DFP^{32}$, but the slope was now less steep, suggesting a different type protein-binding. Identical results were found when human red cells were incubated at 37° C. for 1 hour. Similar saturation levels and slopes were obtained with rabbit and rat cells.

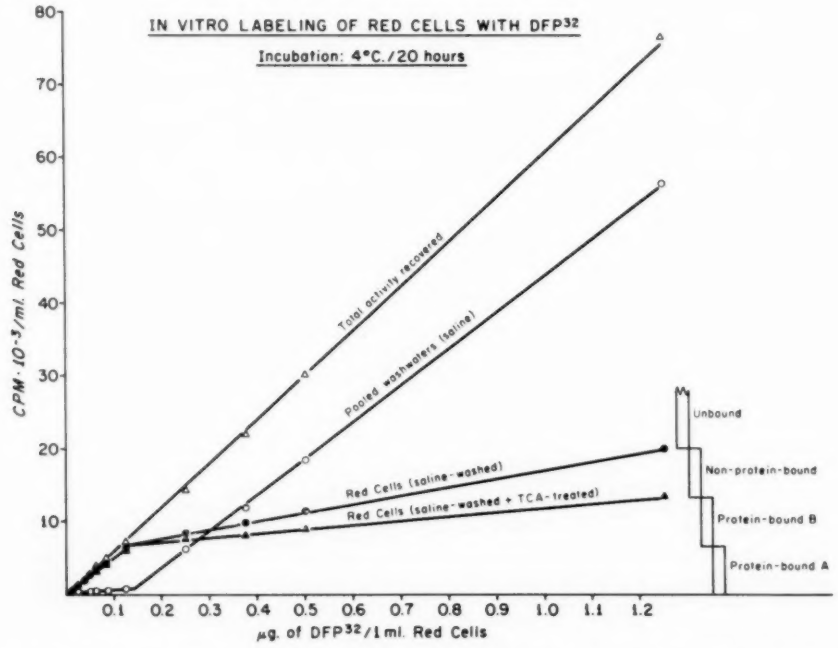


Fig. 1.—In vitro labeling of human red cells with $DFP^{32}$. The total activity recovered is the sum of the activity of the pooled washwaters and the activity of the red cells.

*Evidence for Red Cell Saturation In Vivo.*—One group of rats was prepared by 2 intramuscular injections of nonradioactive DFP (400 μg per kilogram) 13 and 7 days prior to injection of radioactive $DFP^{32}$ (180 μg per kilogram). The red cell uptake of radioactivity by this loaded group was compared to a normal group (Fig. 2). Despite similar hematocrits in the 2 groups (40

and 46.6), the uptake was 5 to 6 times greater in the unloaded group. This indicates that the loaded group had only 17 to 20 per cent of the normally available binding sites. The rat's red cell life span is estimated to be 60 days,[2] so that 12 per cent new cells should have been formed after the last injection of nonradioactive DFP. Therefore, the nonradioactive DFP very nearly saturated red cell binding sites at the time of its injection.

*Early Loss of Label After In Vivo Injection.*—In both groups of rats (reported in previous paragraph) there was an initial drop of approximately 50 per cent in red cell radioactivity during the first few days after in vivo labeling. This drop could not be explained by red cell destruction, since the hematocrit was stable. Nor could it be explained by contamination with white cells or platelets, since platelets carry only about 0.3 per cent,[5] and white cells about 0.5 per cent,[9] of the total radioactivity in blood shortly after the injection of $DFP^{32}$. Therefore, the drop must be due to a loss of label from the red cells. Four fifths of this loss as determined by TCA precipitation was protein-bound $DFP^{32}$.

The early loss following intramuscular injection of large doses in rats lasted for about 5 days, and about 75 per cent of the 1 hour activity was lost during this period (Fig. 3).

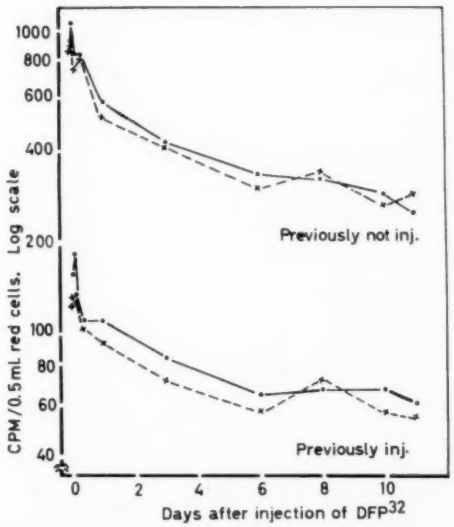


Fig. 2.—The effect of preliminary loading with nonradioactive DFP: red cell radioactivity after intramuscular injection of $DFP^{32}$ in 2 groups of rats, one of which had been previously loaded with nonradioactive DFP. Each point represents 1 rat. Semilogarithmic paper is used to facilitate comparison of the 2 groups.
●——● = radioactivity of washed red cells.
×- - - -× = radioactivity of washed and TCA-treated red cells.

To determine whether this loss of label could be avoided by using a very small dose, 32 $\mu$g, representing about 5 $\mu$c $DFP^{32}$, was injected intravenously into 2 human subjects. Five minutes later, 16 and 17 per cent of the injected dose was in circulation, and 82 and 86 per cent of this was carried by the red cells. (These calculations are based on an assumed blood volume of 5,000 ml.)

Fig. 4 shows that there was a loss of about 20 per cent of the activity during the first 24 hours from washed red cells, and about 15 per cent from their precipitated proteins. Thus, the loss is smaller and lasts for a shorter time, but cannot be avoided.

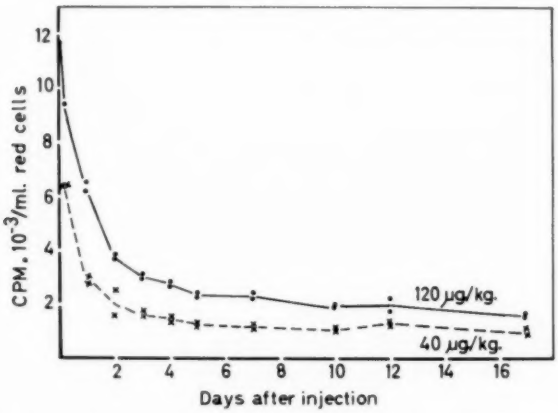


Fig. 3.—Radioactivity of washed red cells after intramuscular injection of $DFP^{32}$ in rats. Each point represents 1 rat.

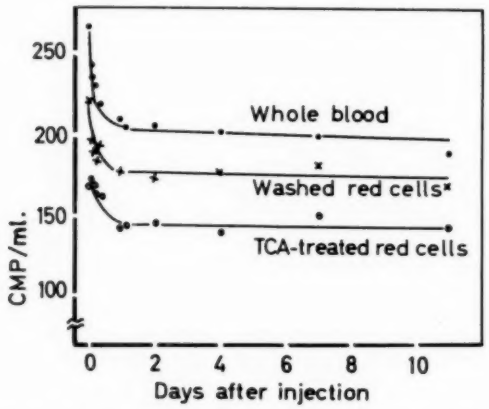


Fig. 4.—Intravenous injection of a small dose (32 µg or 5 µc) of $DFP^{32}$ into a healthy volunteer.

*Transfusion of Red Cells Tagged In Vitro With $DFP^{32}$.*—Rabbit erythrocytes were incubated overnight at 4° C. with 10 times the saturation dose of $DFP^{32}$. The following morning, 90 per cent of the activity was removed by washing, so that the amount transfused corresponded approximately to the in vitro saturation level. The cells were also tagged with $Cr^{51}$ before transfusion. During the first 21 hours after transfusion the $Cr^{51}$ activity dropped to 80 per cent of the 5 minute value, whereas the $DFP^{32}$ activity dropped to 52 per cent (Fig. 5). These studies indicate that there may be some red cell destruction after incubation with high levels of $DFP^{32}$, but the loss of $DFP^{32}$ was far in excess of the loss of red cells as determined by the chromium tag.

A similar study was performed in man, using the smallest possible amount of $DFP^{32}$ per milliliter red cells. The red cells from one unit (450 ml.) of blood were incubated for 1 hour with $DFP^{32}$ in a concentration of 0.1 to 0.3 $\mu$g per milliliter red cells and with $Cr^{51}$ in a concentration of less than 0.10 $\mu$g

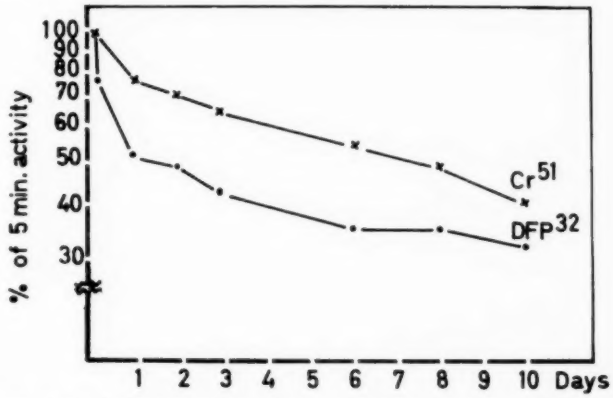


Fig. 5.—Transfusion of washed rabbit red cells labeled in vitro with 1.2 $\mu$g $DFP^{32}$ and 0.08 $\mu$g $Cr^{51}$ per milliliter packed cells. The curves represent the activity of washed red cells.

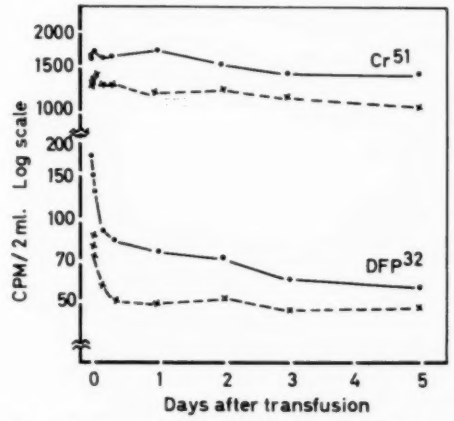


Fig. 6.—Transfusion of washed human red cells labeled in vitro with 0.30 $\mu$g $DFP^{32}$ and 0.004 $\mu$g $Cr^{51}$ per milliliter packed cells.
○——○ = radioactivity of washed red cells.
×- - - -× = radioactivity of washed and TCA-treated red cells.

per milliliter red cells. About 30 to 60 per cent of the $DFP^{32}$ and 5 per cent of the $Cr^{51}$ activity were lost in the wash. The washed cells were transfused back into the donors, and the radioactivity of the red cells followed during 8 days. The transfused cells showed an early loss of $DFP^{32}$ in excess of the $Cr^{51}$ loss. Fig. 6 shows an experiment with 0.30 $\mu$g $DFP^{32}$ per milliliter red cells. The cells lost 40 to 50 per cent of their $DFP^{32}$ activity during the first day. There was no detectable loss of $Cr^{51}$. When only 0.10 $\mu$g $DFP^{32}$ was used per milliliter red cells, the early loss in $DFP^{32}$ was about 20 per cent.

*Injection of Hydrolyzed $DFP^{32}$ ($DIP^{32}$).*—One possible explanation for the early loss of label would be that part of the $DFP^{32}$ preparation had been

hydrolyzed to $DIP^{32}$. To test this possibility, $DIP^{32}$ was injected intraperitoneally in rats in doses corresponding to 130 $\mu$g $DFP^{32}$ per kilogram. The rats were killed at intervals, and their red cells were examined for radioactivity. As shown in Fig. 7, the saline-washed red cells contained detectable radioactivity for 8 hours after the injection, but the TCA-treated cells were free of activity. Similar results were found in rabbits. These experiments suggest that the protein-bound radioactivity in previous experiments was originally present in the preparation as $DFP^{32}$.

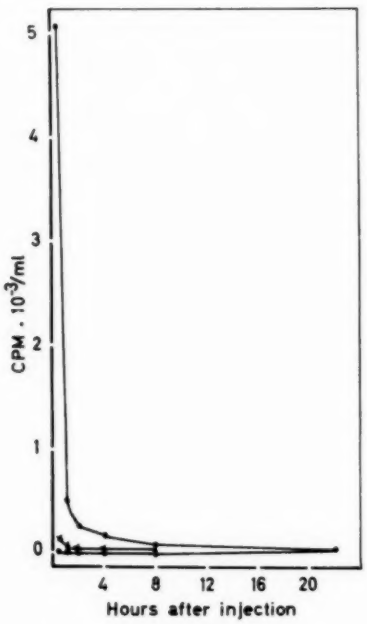


Fig. 7.—Injection of hydrolyzed $DFP^{32}$ ($DIP^{32}$) into rats. The equivalent of 130 $\mu$g $DFP^{32}$ was injected intraperitoneally.
●——● = radioactivity of whole blood.
×——× = radioactivity of washed red cells.
○——○ = radioactivity of washed and TCA-treated red cells.

## DISCUSSION

$DFP^{32}$ is considered to be irreversibly bound to proteins. Elution is, therefore, thought to be impossible,[8, 10-13] and several investigators found no indication for elution from red cells.[12, 14, 15] However, Bove and Ebaugh,[1] and van Putten,[2] have recently reported an unexplained early drop in the red cell life span curve. We have confirmed this, and have shown that the drop is due to a release of label from the cells, i.e., to elution.

Our in vitro experiments showed that red cells have a limited capacity for carrying $DFP^{32}$, and 0.14 $\mu$g per milliliter packed red cells appears to be the saturation dose. The uptake was about 90 per cent with this dose. The in vitro uptake depends on the dose of $DFP^{32}$ and on the treatment of the cells before assay (Fig. 1). The different uptakes reported in the literature,[1, 14] are, therefore, not at variance with our findings.

Elution might be explained by at least 4 different mechanisms:

1. *Overloading.*—Since the in vitro experiments showed that red cells could be overloaded, we first thought that elution was always due to this mechanism. However, elution takes place even after extremely small doses of $DFP^{32}$, and the results with the TCA-treated cells confirmed that some of the protein-bound label was lost. Therefore, elution is not due to overloading only, and it cannot be avoided by using small doses. However, overloading leads to different types of binding and to increased elution. In agreement with this rule, Bove and Ebaugh[1] found that 90 per cent of the activity (of saline-washed red cells) disappeared in 24 hours after transfusion of red cells labeled in vitro with 2 $\mu$g $DFP^{32}$ per milliliter blood. The risk of overloading is great when cells are labeled in vitro, when large doses are injected, and when preliminary blocking doses of nonradioactive DFP are used.

2. *Radioactive Contaminations.*—$P^{32}$ was the only radioactive material present in the $DFP^{32}$ preparation, since the decay curve corresponded exactly to the expected curve for $P^{32}$. Inorganic phosphorus was probably not present, since the last step in the production of $DFP^{32}$ is a distillation. Several organic phosphorus compounds might be present, but this is not a likely explanation, since hydrolyzed $DFP^{32}$ failed to label red cells (Fig. 7). We also observed elution after injections of small doses of $DFP^{32}$ preparations in which the total phosphorus corresponded closely to the amount of phosphorus to be expected from the $DFP^{32}$ content. Therefore, elution is probably not due to contamination with non-$DFP^{32}$ radioactive compounds. However, the possibility of contamination calls for better standardization of the $DFP^{32}$ preparations, and we have recently discussed this problem.[16] To avoid overloading, it is necessary to have well standardized preparations.

3. *Protein Absorption on Red Cells.*—The results could be explained by the hypothesis that $DFP^{32}$ is in part attached to a protein on the red cell surface, which has a more rapid turnover than the red cell itself. This would not be true elution and would be compatible with the assumption that the binding of DFP to proteins is irreversible. From a practical point of view, however, this may be considered elution of label from the cells.

4. *Fluorophosphatase.*—The label might be split off by a specific mechanism, and the red cells do contain small amounts of this enzyme.[17] If this should be the mechanism of elution, it is difficult to see why the elution would stop after a few days.

We have not been able to elucidate the mechanism of elution, but the practical conclusion is that $DFP^{32}$ does elute from the red cells during a short period after labeling. Because of this elution, $DFP^{32}$ should not be used for studies of red cells with rapid turnover, since the results would then be grossly influenced by elution. For red cells with a more normal turnover, however, the early elution will not disturb the results, since there is probably no elution later on.[1, 2]

## SUMMARY AND CONCLUSIONS

1. In vitro experiments show that red cells can carry only a limited amount of radioactive diisopropylfluorophosphate ($DFP^{32}$). When the dose exceeds 0.14

$\mu$g $DFP^{32}$ per milliliter packed human red cells, the uptake is incomplete and there are apparently several types of binding.

2. After injection of $DFP^{32}$ the red cell activity drops rapidly during an initial period, followed by a slow and constant disappearance. The early drop is caused by a loss of both protein-bound and nonprotein-bound label from the cells. This elution takes place even after very small doses of $DFP^{32}$ (32 $\mu$g in humans).

3. Red cells were labeled in vitro with small amounts of $DFP^{32}$ and $Cr^{51}$ and were then transfused back into the donors. The early drop in $DFP^{32}$ greatly exceeded the drop in $Cr^{51}$, an indication that $DFP^{32}$ was lost by elution.

4. The mechanism of elution is unknown, but it is not due to radioactive contaminations. It is greater after large doses of $DFP^{32}$, probably because of overloading.

5. Because of elution, the results are erratic during the first 1 to 3 days, and $DFP^{32}$ should not be used as a red cell tag when this period is important.

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# GLYCOLYTIC, CITRIC ACID CYCLE, AND HEXOSEMONOPHOSPHATE SHUNT ENZYMES OF PLASMA AND ERYTHROCYTES IN MEGALOBLASTIC ANEMIA

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PREVIOUS studies in this laboratory[1, 2] have shown that patients with megaloblastic anemia have elevated serum levels of lactic dehydrogenase and normal levels of glutamic oxaloacetic transaminase. It was assumed that the source of the increase of lactic dehydrogenase in the serum is the erythrocyte and its precursors in the characteristically hypercellular megaloblastic bone marrow. An increased enzyme content of the megaloblastic cells was considered to be possible in view of the known increased glycolytic activity of immature and neoplastic cells.[3, 4] It appeared to be of interest to study other enzymes of carbohydrate metabolism in the serum or plasma, the red blood cells, and the bone marrow of patients with megaloblastic anemia.

Representative enzymes of the glycolytic pathway, citric acid cycle, and the hexosemonophosphate shunt were selected for analysis. Of the 6 enzymes studied, lactic dehydrogenase (LD), malic dehydrogenase (MD), and 6-phosphogluconic dehydrogenase (6-PGD) showed the highest plasma levels, while the levels of isocitric dehydrogenase (ICD), aldolase (Ald.), and phosphohexoisomerase (PHI) were increased to a lesser degree. The peripheral erythrocytes showed only minor abnormalities in enzyme content. The bone marrow analysis was uninformative because of technical difficulties.

## METHODS AND MATERIAL

Sixteen patients with megaloblastic anemia in relapse were examined. All had anemia at the time of initial study. The red cell concentration ranged between 800,000 and 2,400,000 per cubic millimeter, hematocrit measurements between 9 and 26 per cent, and hemoglobin values between 4 and 9.5 Gm. The bone marrow was megaloblastic in all patients. The enzyme determinations were performed before therapy was started. In 7 patients the determinations were repeated during the recovery phase. The diagnosis of Addisonian anemia was made in 7 patients on the basis of the demonstration of the characteristic defect in absorption of $Co^{60}$-cyanocobalamin; in 2 additional patients this diagnosis was established from the clinical data, but absorption studies were not performed. One patient had a total gastrectomy because of carcinoma; 2 had the "malabsorption syndrome"; in 3 patients the megaloblastic anemia was associated with severe malnutrition and chronic alcoholism; 1

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patient had the rare combination of megaloblastic anemia, sickle cell-thalassemia, and a transient extracorpuscular hemolytic process. This patient, who also had severe alcoholism, was observed in relapse on 2 occasions, and has been the subject of a separate report.[5]

Platelet-poor plasma was prepared from venous blood rendered noncoagulable by 10 per cent EDTA (0.15 ml. per 10 ml. of blood). The blood was centrifuged for 30 minutes at 2 to 5° C. and 1,400 g. A red cell sample, minimally contaminated with leukocytes or platelets, was obtained by careful pipetting from the lower half of the packed erythrocytes. The cells were washed 3 times with physiologic saline; after the last wash, a 0.5 ml. aliquot of the packed cells was resuspended in 4.5 ml. of physiologic saline solution and was counted. Hemolysis was produced by freezing and thawing. The sample was then homogenized in a Potter-Elvehjem homogenizer, and the stroma was removed. No enzyme activity could be detected in the stroma. The majority of the samples were examined immediately. An occasional one was frozen at −10° C. and studied several days later.

In a few samples, as indicated in Table III, the enzyme activity was determined in serum as it had been done in our previous study.[1, 2] Subsequently, in our laboratory, the enzyme values in plasma have been found to be somewhat lower than in serum (Table I),[6] and the standard deviations of the normal mean greater in serum than in plasma. Because of these observations platelet-poor plasma was considered preferable for the enzyme analysis of this study.

The enzyme determinations were performed according to standard methods with minor modifications. The enzyme values in the plasma were expressed as millimicromoles of one of the reaction components per milliliter per minute. All reactions took place at 37° C.

For the measurement of lactic dehydrogenase the method of Neilands[7] was adapted to plasma and hemolysates. A 3.0 ml. reaction system in 1 cm. cuvettes was employed. A previously described[2] modification of Neilands' method was used for the determination of this enzyme in the serum of 3 patients (Table III). The method of Ochoa[8] was used for the determination of malic dehydrogenase. For the measurement of 6-phosphogluconic dehydrogenase and isocitric dehydrogenase the methods described by Wolfson and Williams-Ashman[9] were employed. The micromethod of Lowry[10] was modified to permit the determination of aldolase activity in 0.1 ml. of the enzyme source.[11] Bodansky's method[12] was used for the determination of phosphohexoisomerase. The activity of this enzyme was expressed as millimicromoles of fructose-6-phosphate per milliliter per minute.

## RESULTS

The normal mean values for plasma and erythrocyte concentration of enzymes are listed in Tables I and II. The results were considered abnormal only when they fell beyond 3 standard deviations of the normal mean value.

*Enzymes in Plasma.—*

*General:* The levels of the 6 enzymes determined were elevated in most or all patients studied. Most marked were the elevations of LD, MD, and 6-PGD. Levels of ICD, Ald., and PHI were less strikingly and less consistently elevated (Table III, Fig. 1). In the 7 patients whose plasma enzymes were determined after initiation of therapy, diminution of the enzyme values was observed (Fig. 2) in most cases.

*Lactic dehydrogenase:* The level of this enzyme was found to be significantly increased in all 12 patients in whom the plasma level was determined and in the 3 patients in whom the serum values were obtained. The highest level was more than 60 times the normal mean. The enzyme level diminished in 5 patients after initiation of therapy. In the patient with sickle cell thalassemia, who also had a transient extracorpuscular hemolytic process, the value

remained abnormal throughout the period of study (120 days). At the time the second value was determined he was recovering from viral hepatitis.

*Malic dehydrogenase:* The plasma level of this enzyme in all patients was above the upper limit of normal. One value was 400 times the normal mean. The other values were distributed between 2½ and 46 times the normal. In 5 patients whose plasma was tested after initiation of therapy, marked reduction of the plasma content was noted (Fig. 2). In only 2 the levels remained slightly increased; one of these was the patient with the sickle cell thalassemia.

*6-phosphogluconic dehydrogenase:* In all 11 patients whose plasma was tested for this enzyme the level was significantly elevated, ranging from three- to twenty-two-fold increases. In 4 out of 5 patients the levels became normal with recovery. In one patient, tested 5 days after start of therapy, the enzyme level remained slightly elevated.

*Isocitric dehydrogenase:* The ICD plasma level was determined in only 8 patients during relapse. In 4 patients it was significantly elevated. The highest level was only 5 times the normal mean. During the recovery phase the enzyme level remained slightly abnormal in only 1 patient.

*Aldolase:* The level of this enzyme was determined in all 16 patients. In 12 patients it was significantly elevated. The highest level was 17 times the normal mean. In 6 of the patients who were tested after start of therapy the level became normal with recovery; in 1 it remained slightly elevated.

TABLE I. MEAN ENZYME VALUES IN PLASMA* AND SERUM* OF 32 NORMAL SUBJECTS

| ENZYME | PLASMA (±1 S.D.)† | SERUM (±1 S.D.)† |
|---|---|---|
| Lactic dehydrogenase (LD) | 82.0 (±19) P <0.001 | 105.0 (±29) mμM DPN.H/ml./min. |
| Malic dehydrogenase (MD) | 52.0 (±12) P <0.001 | 79.0 (±38) mμM DPN/ml./min. |
| 6-Phosphogluconic dehydrogenase (6-PGD) | 1.6 (±0.8) P <0.001 | 3.2 (+2) mμM TPN.H/ml./min. |
| Isocitric dehydrogenase (ICD) | 2.5 (±1.6) P <0.001 | 5.7 (±2.9) mμM TPN.H/ml./min. |
| Aldolase (Ald.) | 1.8 (±0.7) P <0.05 | 2.4 (±0.9) mμM F-1.6-P/ml./min. |
| Phosphohexoisomerase (PHI) | 30.0 (±14) P <0.001 | 54.0 (+25) mμM F-6-P/ml./min. |

*EDTA (0.15 ml. of a 10 per cent solution per 10 ml. of blood) was used as anticoagulant. An equal amount was added to serum.[6]

†Values were calculated by the method of Student.

TABLE II. MEAN ENZYME VALUES IN ERYTHROCYTES OF 20 NORMAL SUBJECTS

| ENZYME | PER $10^6$ ERYTHROCYTES (±1 S.D.) | PER MG. HGB.* (±1 S.D.) |
|---|---|---|
| LD | 1.9 (±0.5) | 60.0 (±18) mμM DPN.H/min. |
| MD | 5.0 (±0.6) | 160.0 (±24) mμM DPN/min. |
| 6-PGD | 0.1 (±0.025) | 3.0 (±0.8) mμM TPN.H/min. |
| ICD | 0.016 (±0.01) | 0.5 (±0.2) mμM TPN.H/min. |
| Ald. | 0.04 (±0.01) | 1.2 (±0.36) mμM F-1,6-P/min. |
| PHI | 0.9 (±0.23) | 30.0 (±6.9) mμM F-6-P/min. |

*Hemoglobin determined in hemolysate with the method of Drabkin.[13]

TABLE III. PLASMA ENZYMES IN MEGALOBLASTIC ANEMIA IN RELAPSE

| SUBJECT | HEMATOCRIT | CAUSE OF ANEMIA | LD | MD | 6-PGD | ICD | ALD. | PHI |
|---|---|---|---|---|---|---|---|---|
| Normal | | | 82 (±19) | 52 (±12) | 1.6 (±0.8) | 2.5 (±1.6) | 1.8 (±0.7) | 30 (±14) |
| Ke | 17.0 | Pernicious anemia | 1,534 | 22,000 | 12.8 | — | 6.7 | 144 |
| Wal | 16.0 | Pernicious anemia | 700 | 215 | — | — | 8.1 | 62 |
| Ta | 9.0 | Malnutrition* | 4,950 | 2,400 | 18.0 | — | 30.0 | 278 |
| Ta | 22.0 | Malnutrition* | 980 | 520 | 7.8 | — | 8.4 | 179 |
| Na | 19.0 | Pernicious anemia | 786† | 1,100 | — | — | 5.3 | 195 |
| Tu | 10.0 | Pernicious anemia | 2,890 | 720 | 35.0 | 3.4 | 13.2 | 300 |
| Bra | 9.5 | Malabsorption | 1,540 | 2,400 | 14.0 | 4.8 | 24.3 | 70 |
| Wat | 20.0 | Pernicious anemia | 460 | 280 | 19.0 | 11.0 | 6.4 | 12 |
| Wi | 22.5 | Pernicious anemia | 455 | 235 | 5.8 | 6.4 | 2.8 | 37 |
| Pi | 12.0 | Total gastrectomy | 673† | 178 | — | — | 6.3 | — |
| Pia | 26.0 | Pernicious anemia | — | 222 | — | — | 4.2 | — |
| Ea | 26.0 | Malnutrition | 455 | 125 | 13.0 | 12.0 | 2.8 | 264 |
| Bro | 18.0 | Malnutrition | 360 | 168 | 9.0 | 9.3 | 1.8 | 56 |
| Atk | 19.0 | Probable pernicious anemia | 1,200 | 505 | 15.0 | 10.0 | 6.7 | 33 |
| Co | 16.0 | Probable pernicious anemia | 820 | 934 | 15.0 | 4.8 | 9.8 | 43 |
| De | 25.0 | Malabsorption | 452† | 251 | — | — | 3.2 | — |

*This patient was observed during two relapses. The megaloblastic anemia was associated with malnutrition, sickle cell thalassemia, and an extracorpuscular hemolytic process.

†Performed in serum by method described in reference.[1] (Normal 186 μM [± 42]/100 ml./30 min.)

*Phosphohexoisomerase:* The level of this enzyme was measured in the plasma of 13 patients. The values ranged from 12 to 300 mμM of F-6-P per milliliter per minute. It was significantly elevated in only 6 patients. The maximal elevation was 9 times the normal mean. The enzyme level was found to be normal during the phase of recovery in 5 patients. A significant rise was observed 5 days after the start of therapy in 1 patient, although the levels of the other enzymes had declined.

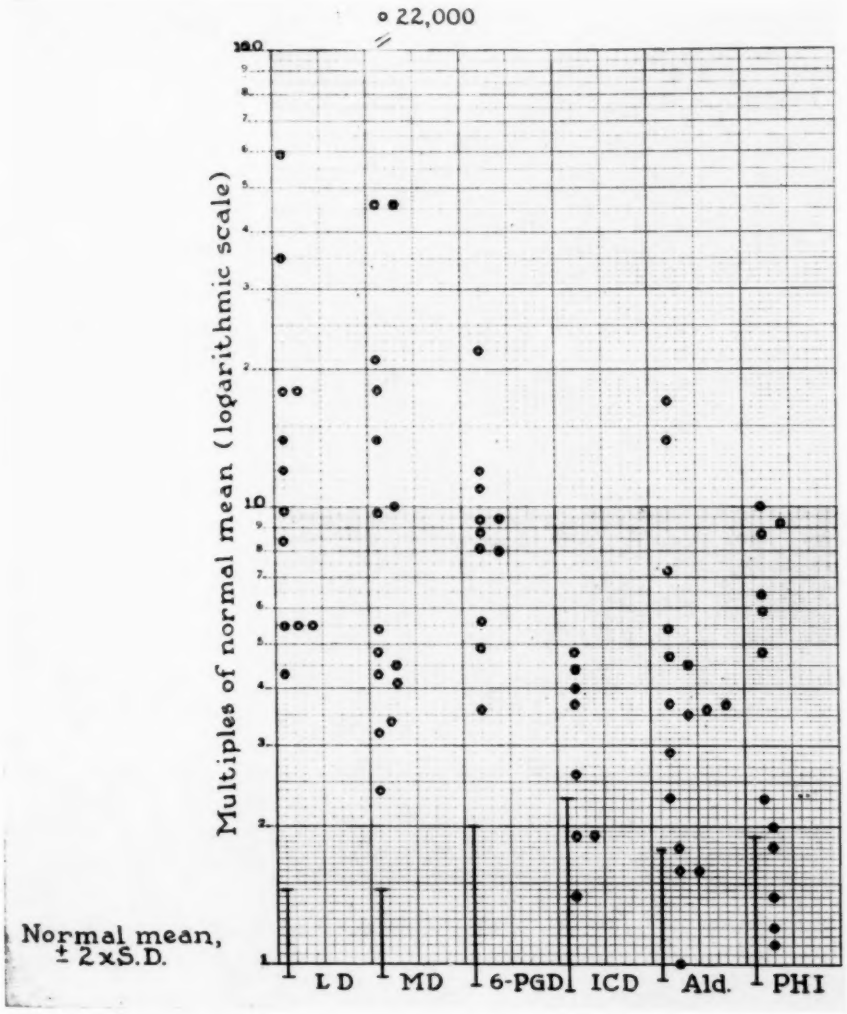


FIG. 1.—Plasma enzymes in megaloblastic anemia in relapse, plotted as multiples of normal mean ±2×S.D. (logarithmic scale).

*Erythrocytes.*—Only minor abnormalities were detected in the enzyme content of the erythrocytes (Table IV). The values for MD showed the greatest deviation from the normal. Nine of 16 patients tested had increased values,

TABLE IV. ERYTHROCYTE ENZYMES IN MEGALOBLASTIC ANEMIA IN RELAPSE

| PATIENT | HEMATOCRIT | CAUSE OF ANEMIA | LD (PER $10^6$ ERYTHROCYTES) | MD (PER $10^6$ ERYTHROCYTES) | 6-PGD (PER $10^6$ ERYTHROCYTES) | ICD (PER $10^6$ ERYTHROCYTES) | ALD. (PER $10^6$ ERYTHROCYTES) | PHI (PER $10^6$ ERYTHROCYTES) |
|---|---|---|---|---|---|---|---|---|
| Normal | | | 1.9 (±0.5) | 5.0 (±0.6) | 0.1 (±0.025) | 0.066 (±0.01) | 0.04 (±0.06) | 0.9 (±0.23) |
| Ke | 17.0 | Pernicious anemia | 4.0* | 5.0 | — | — | 0.04 | 0.2 |
| Wal | 16.0 | Pernicious anemia | 1.8 | 9.8* | — | — | 0.07 | 0.04 |
| Ta | 9.0 | Malnutrition | 4.0* | 12.0* | — | — | 0.07 | 2.4* |
| Ta | 22.0 | Malnutrition | 0.9 | 7.2* | — | — | 0.04 | 2.1* |
| Na | 19.0 | Pernicious anemia | 2.5 | 8.7* | — | — | 0.02 | 1.7* |
| Tu | 10.0 | Pernicious anemia | 5.2* | 10.0* | 0.17 | 0.02 | 0.04 | 2.2* |
| Bra | 9.5 | Malabsorption | 3.8* | 15.2* | 0.20 | 0.01 | 0.03 | 1.9* |
| Wat | 20.0 | Pernicious anemia | 3.2 | 6.4 | 0.19* | 0.026 | 0.04 | 1.1 |
| Wi | 22.5 | Pernicious anemia | 4.1* | 8.0 | 0.17 | 0.065 | 0.06 | 0.9 |
| Pi | 12.0 | Total gastrectomy | 1.7 | 7.8* | — | — | 0.04 | 0.7 |
| Pia | 26.0 | Pernicious anemia | — | 5.8 | — | — | 0.03 | 1.0 |
| Ea | 26.0 | Malnutrition | 1.4 | 4.3 | 0.11 | 0.02 | 0.02 | 0.4 |
| Bro | 18.0 | Malnutrition | 2.7 | 4.7 | 0.11 | 0.03 | 0.03 | 0.4 |
| Atk | 19.0 | Probable pernicious anemia | 2.0 | 4.1 | 0.08 | 0.024 | 0.02 | 1.0 |
| Co | 16.0 | Probable pernicious anemia | 2.1 | 3.8 | 0.07 | 0.013 | 0.02 | 0.6 |
| De | 25.0 | Malabsorption | — | 14.0* | — | — | 0.06 | — |

*Abnormal values (> normal mean + 3 S.D.).

the highest of which was 7 standard deviations above the normal mean. The next most frequent abnormality was observed with LD; 5 values were slightly abnormal. The erythrocyte level of 6-PGD was found to be minimally elevated in only 1 of 8 patients tested. Ald. and ICD were normal in all patients tested. Five of 15 patients tested had minimal PHI elevations. When the enzyme content was calculated per milligram of hemoglobin, these values tended to be less abnormal because of the macrocytosis.

In 6 patients the enzyme content of the red cells was determined during the recovery phase. Except for 2 MD values and 1 LD value, which remained slightly elevated, results of these determinations were normal.

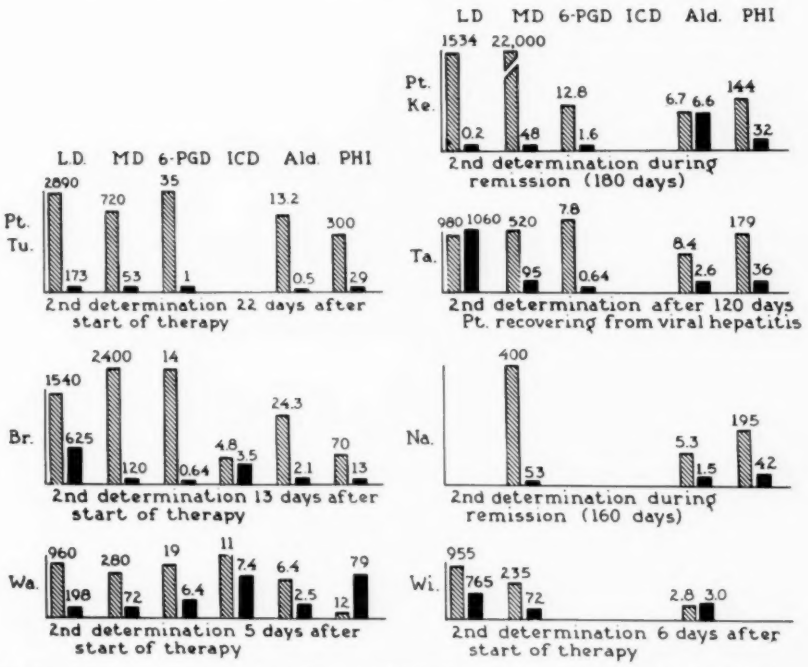
LD MD 6-PGD ICD Ald. PHI
Pt. Tu.
2890 720 35 13.2 300
173 53 1 0.5 29
2nd determination 22 days after start of therapy
LD MD 6-PGD ICD Ald. PHI
Pt. Ke.
1534 22,000 12.8 6.7 6.6 144
0.2 48 1.6 32
2nd determination during remission (180 days)
Ta.
980 1060 520 7.8 8.4 179
95 0.64 2.6 36
2nd determination after 120 days
Pt. recovering from viral hepatitis
Br.
1540 2400 14 24.3 70
625 120 0.64 4.8 3.5 2.1 13
2nd determination 13 days after start of therapy
Na.
400 5.3 195
53 1.5 42
2nd determination during remission (160 days)
Wa.
960 280 19 11 7.4 6.4 79
198 72 6.4 2.5 12
2nd determination 5 days after start of therapy
Wi.
955 235
765 72 2.8 3.0
2nd determination 6 days after start of therapy

Fig. 2.—Plasma enzyme values in megaloblastic anemia before and after treatment.

## DISCUSSION

Elevated levels of LD were observed in the plasma of all patients with megaloblastic anemia in relapse. This is consistent with the previous reports on serum LD levels from this[2] and other laboratories.[14, 15] Recently, Levitan, Wasserman, and Wroblewski[16] have also reported elevated levels of LD in the serum of patients with pernicious anemia. These workers, however, did not observe elevated serum levels of this enzyme in other types of megaloblastic anemia. This is at variance with our observations of elevated enzyme levels in megaloblastic anemia of varied etiology. Gordon and Enari[14] also have reported LD elevations in the serum of patients with megaloblastic anemia caused by infestation with *Diphyllobothrium latum*.

In the present study it has been shown that the plasma levels of 2 other glycolytic enzymes also are elevated, but not as markedly or as consistently as the LD. Of 2 enzymes of the citric acid cycle studied in the plasma the levels of MD were high, and the elevations of ICD were less marked. The level of the hexosemonophosphate shunt enzyme 6-PGD was also markedly elevated.

The high plasma enzyme levels usually decreased rapidly after therapy had been started (Fig. 2). This phenomenon followed closely the prompt conversion of megaloblastic into normoblastic erythropoiesis, and occurred before the hemoglobin and erythrocyte levels had risen significantly. This sequence of events suggests that the high enzyme levels in the plasma during the fully developed megaloblastic state might reflect increased enzyme content and the abnormal metabolism of the megaloblastic bone marrow. The high intramedullary destruction rate, which is a characteristic feature of the megaloblastic maturation arrest[17] and accounts for the ineffective erythropoiesis despite the increased proliferative activity of the bone marrow, probably leads to the release of intracellular enzymes into the surrounding plasma.

The problem of the metabolic abnormalities in the megaloblastic precursor cells might best be investigated by studies of enzyme content or metabolic activity of the bone marrow. Our attempts to determine enzyme concentration in the nucleated cells of the bone marrow were unsuccessful for technical reasons. However, some indirect evidence bearing on this question is available. Rubinstein, Ottolenghi, and Denstedt[18] have shown that the mature mammalian erythrocyte is deficient in citric acid cycle enzymes and incapable of respiratory activity. The immature cells, however, including the reticulocytes, contain mitochondria and utilize the citric acid cycle in their metabolic activity. While these authors found 6-PGD activity in the mature erythrocyte and the reticulocyte of the rabbit, Marks[19] has reported that, in man, the activity of this enzyme and of glucose-6-phosphate dehydrogenase is higher in reticulocytes and in the younger erythrocytes than in the aged cells. It is of interest that in the present study the intraerythrocytic levels of 6-PGD, ICD, Ald., and PHI were shown to be normal in most cases. This might be explained by the predominance of aged cells and the reticulocytopenia in the peripheral blood. The frequent MD and LD elevations could be caused by the very high enzyme levels in the plasma. It is conceivable that a minimal amount of plasma remained attached to the red cells, despite repeated washing of the cells prior to hemolysis. This requires further study.

The decreased survival rate of the peripheral cell which has been demonstrated in megaloblastic anemia[17, 20] may also contribute to the enzyme elevations in plasma, but it should be emphasized that, in other hemolytic states, the enzyme elevations are only mild to moderate[2, 21] and do not reach the peaks of those in megaloblastic anemia. It seems likely that the more important source of the high plasma levels is the bone marrow.

The factors responsible for the varying degrees of plasma activity of the 6 enzymes studied in any patient are obscure. ICD has not been observed to deviate as much from the normal plasma levels as MD, the other citric acid

cycle enzyme tested. Similarly, LD levels are distinctly higher than those of PHI and Ald. These differences might be ascribed to variations in clearance, inhibition, or potentiation. It seems probable that enzymes, once they are released into the plasma, are subject to different and perhaps specific ways of disposition.

## SUMMARY

1. The plasma of patients with megaloblastic anemia in relapse was found to contain markedly increased levels of lactic, malic, and 6-phosphogluconic dehydrogenase. The activity of phosphohexoisomerase, aldolase, and isocitric dehydrogenase was increased less consistently and to a lesser extent.

2. Occasional minor increases in intraerythrocytic enzyme activity of malic, lactic, and 6-phosphogluconic dehydrogenases were found in the peripheral red cell.

3. All these abnormalities tended to disappear promptly with the conversion of the megaloblastic into normoblastic erythropoiesis.

4. These alterations occurred independently of the etiology of the megaloblastic anemia.

5. It is postulated that these plasma abnormalities are the result of increased enzyme content of the megaloblastic cell and the increased intramedullary turnover rate of the megaloblasts, associated with some increase in the peripheral destruction rate.

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# THE EFFECT OF HEPARIN AND EDTA ON DNA SYNTHESIS BY MARROW IN VITRO

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HEPARIN and ethylenediaminetetraacetic acid (EDTA) are widely used in clinical and laboratory medicine. The effect of these anticoagulants on cell division and cell viability is largely unknown or controversial.

EDTA inhibits mitosis of plant cells in vitro,[1, 2] but its effects on mammalian cell division have not been extensively documented. The effect or lack of effect of heparin on cell division has been the subject of investigation for years. Fischer,[3] utilizing heart fibroblasts, found that heparin in concentrations of 0.05 per cent prevented cell division in vitro. Heilbrunn[4] found that this same concentration of heparin inhibited egg cell division in vitro. Goerner[5] showed that in vitro exposure to 0.1 per cent heparin rendered a Flexner-Jobling carcinoma nontransplantable in vivo. Lippman[6] found that heparin inhibited mitosis of the Ehrlich ascites tumor in vivo. However, Kreisler,[7] studying a transplantable lymphosarcoma in vivo, and King and co-workers,[8] studying Strain L cells in vitro, found that heparin had no effect on mitosis.

Recently, during the course of investigations directed at transplantation of viable marrow cells in vivo, the question of toxicity from the necessary anticoagulants arose. Because of the lack of agreement as to the effect of heparin on cell division, the paucity of information concerning the effect of EDTA, and the importance of maintaining cell viability in vitro, further investigations seemed warranted.

Techniques for the estimation of inhibition of cell division depend either upon morphologic observations or upon cell count measurements. We have described an in vitro test for desoxyribonucleic acid (DNA) synthesis which depends on incorporation of $C^{14}$ formate into thymine of DNA.[9] Since DNA synthesis is intimately related to cell division, the effect of heparin and EDTA on DNA synthesis in vitro provides information on the effect of these anticoagulants on cell proliferation. This report describes observations on the effect of heparin and EDTA on DNA synthesis by rabbit and human marrow cells in vitro.

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## MATERIALS AND METHODS

Rabbit marrow was obtained from the long bones of normal animals. Human marrow was obtained from ribs removed during a cardiac operation. The technique for the preparation of the marrow, incubation with radioactive formate and determination of the specific activity of the isolated thymine, has already been described.[9]

The $C^{14}$ formate used had a specific activity of 2.3 $\mu c$ per micromole. The incubation flasks contained 2.3 $\mu c$ of $C^{14}$ formate, 250 U. penicillin, and 100 meg. streptomycin.

The heparin used in these experiments was a powdered heparin sodium which contained 110 U. per milligram.* This heparin contained no preservative. The heparin and EDTA† were diluted to the desired concentration with Hanks' solution.

## RESULTS

Table I shows that incubation of rabbit bone marrow cells in concentrations of heparin from 0.025 mg. per milliliter (0.0025 per cent) to 1.0 mg. per milliliter (0.1 per cent) resulted in no significant depression of DNA synthesis. To exclude the possibility that our incubation time had been too short and that aliquots had contained excessive numbers of cells, the experiment illustrated

TABLE I

| FLASK | RABBIT MARROW IN SERUM | HEPARIN* | PHENOL† | THYMINE RADIOACTIVITY‡ |
|---|---|---|---|---|
| | ($90 \times 10^6$ nucleated cells) | | | |
| 1 | | —— | —— | 4,415 |
| 2 | | 0.025 | —— | 4,040 |
| 3 | | 0.05 | —— | 4,040 |
| 4 | | 0.1 | —— | 4,070 |
| | ($151 \times 10^6$ nucleated cells) | | | |
| 5 | | —— | —— | 5,015 |
| 6 | | 0.5 | —— | 5,330 |
| 7 | | 1.0 | —— | 4,450 |
| 8 | | —— | 0.45 | 1,090 |
| 9 | | 0.5 | 0.23 | 2,000 |

Incubation—1 hour at 37.5° C.
*Heparin sodium, 110 U. per milligram.
†Recrystallized phenol (made up to desired concentration in Hanks' solution).
‡Counts per minute per micromol of thymine isolated from marrow DNA (average of duplicate determinations).

TABLE II

| FLASK | RABBIT MARROW IN SERUM | HEPARIN* | THYMINE RADIOACTIVITY† |
|---|---|---|---|
| | ($128.5 \times 10^6$ nucleated cells) | | |
| 1 | | —— | 58,150 |
| 2 | | 0.1 | 63,650 |
| 3 | | 1.0 | 56,500 |
| | ($14.75 \times 10^6$ nucleated cells) | | |
| 4 | | —— | 12,900 |
| 5 | | 0.1 | 9,385 |
| 6 | | 1.0 | 10,825 |

Incubation—22 hours at 37.5° C.
*Heparin sodium, 110 U. per milligram.
†Counts per minute per micromol of thymine isolated from marrow DNA (average of duplicate determinations).

*From Connaught Medical Research Laboratories, Toronto, Canada.
†EDTA was obtained from Platelet Pack, Ethicon, Inc., Somerville, N. J.

in Table II was performed. It can be seen that heparin had no effect on DNA synthesis by either $14.75 \times 10^6$ or $128.5 \times 10^6$ nucleated cells incubated for 22 hours.

Since commercial liquid heparin solutions frequently contain phenol or phenol derivatives in concentrations of 0.2 to 0.45 per cent as preservatives, the effect of small concentrations of phenol on DNA synthesis was studied. It can be seen from Table I that as little as 0.23 mg. per milliliter (0.023 per cent) phenol significantly depressed DNA synthesis. Other experiments, not shown here, revealed no effect from less than 0.12 mg. per milliliter phenol, and no depression in DNA synthesis from the use of 0.01 mg. per milliliter to 0.1 mg. per milliliter of a commercially available heparin solution.* However, higher concentrations of heparin, 3.33 mg. per milliliter, which contained the preservatives listed in the footnote, did inhibit DNA synthesis.

Table III shows that DNA synthesis by human marrow is not influenced by heparin in the concentrations indicated, but is depressed by 0.5 mg. per milliliter (0.05 per cent) EDTA. The DNA synthesis by marrow cells inhibited by EDTA returned to normal when the exposure to EDTA in the concentration listed was less than 3 hours, and when the EDTA was removed from the cells prior to incubation.

## DISCUSSION

The data presented demonstrate that heparin in a concentration range useful for the prevention of coagulation of aspirated marrow does not inhibit DNA synthesis by marrow cells in vitro. Since marrow is composed of a mixture of types of cells, it is possible that heparin could inhibit one type of cell and not another, but a general inhibition of the DNA synthesis necessary for mitosis should, if present, have been apparent in the studies reported here. These in vitro observations are in accord with results that show that marrow, stored at 4° C. for 4 to 48 hours in the presence of heparin (0.1 mg. per milliliter), will divide and repopulate marrow spaces, when introduced in vivo.[10] The fact that other investigators have used impure heparin, and, in some instances, heparin which contained preservatives, and have relied largely on

TABLE III

| FLASK | HUMAN MARROW IN SERUM (NO. NUCLEATED CELLS) | HEPARIN* (MG./ML.) | EDTA† (MG./ML.) | THYMINE RADIOACTIVITY‡ |
|---|---|---|---|---|
| 1 | $235 \times 10^6$ | —— | —— | 9,973 |
| 2 | $235 \times 10^6$ | 1.0 | —— | 12,094 |
| 3 | $235 \times 10^6$ | 0.1 | —— | 10,118 |
| 4 | $235 \times 10^6$ | —— | 0.5 | 4,668 |
| 5 | $235 \times 10^6$ | —— | 0.1 | 8,668 |

Incubation—4 hours at 37.5° C.

*Heparin sodium, 110 U. per milligram.

†One and one-half per cent disodium dihydrogen ethylenediaminetetraacetate dihydrate, ar 0.7 per cent sodium chloride obtained from Platelet Pack, Ethicon, Inc., Somerville, N. J.

‡Counts per minute per micromol of thymine isolated from marrow DNA (average of d icate determinations).

*Heparin sodium, 10 mg. per milliliter, with methylparaben, 0.18 per cent, and propyl- ben, 0.02 per cent, Abbott Laboratories, North Chicago, Ill.

morphologic observations for the estimation of inhibition of mitosis, may explain their conclusions regarding inhibition of mitosis attributed to this anticoagulant.

Kurnick and co-workers[11] referred to a "damaging effect" on marrow cells by excessive concentrations of heparin. The heparin* used contained phenol as a preservative, and, as indicated in Table I, marrow cell division is considerably depressed in the presence of phenol. Heparin with phenol has been used without apparent damage to marrow cells in vitro where the concentration of phenol has not exceeded 0.1 mg. per milliliter (0.01 per cent).

The inhibitory effect of EDTA on DNA synthesis by marrow cells is marked and reproducible. This is consistent with reports in the literature[1, 2] of the ability of this anticoagulant to inhibit plant cell mitosis in vitro. Brief exposure to EDTA did not seem to inhibit marrow DNA synthesis permanently, but the exposure time necessary for irreversible damage is not known, and this anticoagulant has not been used in this laboratory for marrow studies in vitro.

## SUMMARY

The effect of heparin and ethylenediaminetetraacetic acid (EDTA) on desoxyribonucleic acid (DNA) synthesis by rabbit and human marrow cells in vitro has been studied. Heparin in concentrations to 1.0 mg. per milliliter did not cause depression in DNA synthesis, unless phenol or other preservatives were concurrently present. EDTA depressed DNA synthesis in a concentration of 0.5 mg. per milliliter. It is recommended that heparin without preservatives be used as the anticoagulant in marrow transplantation procedures.

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*Heparin sodium U.S.P., 100 mg. per cubic centimeter, Darwin Laboratories, Los Angeles, Calif.

# EXPERIMENTAL HYPERSENSITIVITY IN THE RABBIT: POTENTIATING EFFECT OF PRIOR SENSITIZATION ON RENAL LESIONS PRODUCED BY BOVINE GAMMA GLOBULIN

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IN RECENT years a number of studies of experimental hypersensitivity (serum sickness) in the rabbit have been reported, in which the relatively pure proteins, bovine serum albumin (BSA) and bovine serum gamma globulin (BGG)[1-7] were used. From these studies, the concept has emerged that the lesions of serum sickness are produced by the immunopathogenic properties of certain soluble antigen-antibody complexes formed by reaction of the injected antigen with newly formed, specific antibody.[5-7] It has been observed, however, that the hypersensitivity lesions seen microscopically after injection of BGG, although about equally frequent, are much less severe than those which follow BSA administration.[8] It has not been clarified whether this difference in response is a result of a difference in the cytotoxicity of the soluble complexes produced, or in the metabolism and antigenicity of these proteins.

In the present study, normal rabbits and rabbits sensitized to BGG have been challenged by intravenous injection of this protein, and their course has been followed by serial blood and urine specimens. In the animals sensitized by injection of BGG in adjuvant, a high proportion developed a nephritis characterized by azotemia and a sustained proteinuria. These changes were less frequently observed in animals sensitized with alum-precipitated BGG. Disease seldom developed in the nonsensitized animals. Microscopic study of the organs of animals killed at the height of the proteinuria showed lesions which, with few exceptions, were closely similar to those described from nonsensitized animals challenged with BSA.

## MATERIALS AND METHODS

Female albino rabbits, maintained on Purina Chow with occasional feedings of greens, and weighing 1,400 to 3,400 grams, were used exclusively.

The bovine gamma globulin* was given intravenously as a 10 per cent solution in 0.15 M sodium chloride solution. Before use the solutions were adjusted to pH 7.4 with sodium bicarbonate and sterilized by Seitz filtration. All animals received 10 ml. of the

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solution (1 Gm. of BGG) per kilogram body weight. Freund's adjuvant, consisting of 8.5 parts Bayol F, 1.5 parts Arlacel A, and 10 parts of sterile saline containing BGG, was injected subcutaneously at 5 sites on back, thighs, and forelegs, 1 ml. into each site. Complete adjuvant contained 0.5 mg. of heat-killed tubercle bacilli per milliliter of Bayol F. The BGG content of the adjuvant was 10 mg. per milliliter of final mixture. Alum-precipitated BGG, buffered to pH 7.5 with acetate and containing 2.4 mg. of K Al $(SO_4)_2$ per milligram of BGG, was given intravenously in a daily dose of 1.5 ml. which contained 2.25 mg. of the antigen.

Nonsensitized animals comprised a group of 30 which received BGG in saline intravenously, without prior sensitization to this protein.

The sensitized group was comprised of 63 animals. Forty-six of these were sensitized by injection of BGG in Freund's adjuvant, and 17 by a course of 8 daily intravenous injections of alum-precipitated BGG. The interval between sensitization and challenge by intravenous injection of BGG varied. In all animals sensitized with the alum preparation, BGG was given intravenously 4 days after the last sensitizing injection. In the adjuvant-sensitized group, the interval between adjuvant injection and BGG challenge was 2 days in 12 animals, 4 days in 27, and 6 days in 11. Six animals in the 2-day group, 6 in the 4-day, and 5 in the 6-day received incomplete adjuvant. In the remainder the adjuvant contained tubercle bacilli.

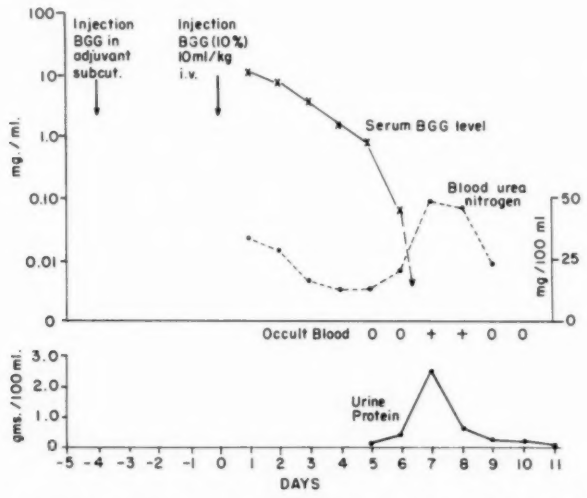


Fig. 1.—Serum BGG levels, blood urea nitrogen, and urine occult blood and protein during the nephritis which follows intravenous challenge with BGG in a rabbit sensitized to BGG. The animal was sensitized by subcutaneous injection of BGG in adjuvant 4 days prior to challenge.

Ten of the animals in the 4-day group were killed for histologic examination of their tissues on or before the first day of proteinuria, and are not included in the statistics which describe the incidence of the disease. A number of others were killed after serial urine examinations definitely identified the presence of the disease.

All of the sensitized animals required desensitization before intravenous administration of BGG. The most extensive desensitization was required for animals challenged with BGG 6 days after Freund's adjuvant, and for those sensitized with the alum-precipitated preparation. Desensitization of these animals consisted of 1 ml. of 0.1 per cent BGG in saline, injected intraperitoneally the evening before the day of challenge, followed on the day of challenge by another 1 ml. of 0.1 per cent intraperitoneally, 1 ml. of 0.1 per cent intravenously, 1 ml. of 1 per cent intravenously, and 5 ml. of 1 per cent intravenously. These injections were given at 2 to 3 hour intervals before injection of the final dose of 10 ml. of

10 per cent BGG per kilogram. Less extensive desensitization consisted of 1 ml. of 0.1 per cent intraperitoneally, 1 ml. of 0.1 per cent intravenously, and 1 ml. of 1 per cent intravenously given at intervals on the day of the final injection.

To avoid catheterization daily urine specimens were obtained by manual pressure on the lower abdomen with the animal in a supine position on an animal board. Urine was tested for occult blood by reaction with a peroxide with orthotolidin as the indicator.* After centrifugation, urine protein was precipitated with Tsuchiya's reagent, the precipitate was dissolved in alkali, and the protein content was determined by the Lowry modification of the Folin-Ciocalteau procedure[9] with bovine serum albumin as the standard. Specimens of blood were obtained from veins in ear or hind leg, and the sera was stored at −20° C. until used. BGG was determined in sera by precipitation with anti-BGG sera by the method described by Germuth for BSA.[3] The precipitates, after a wash, were dissolved in alkali, and their protein content was determined by the Folin-Ciocalteau method.[9] Fresh BGG-anti BGG complex, formed in slight antigen excess, dissolved in alkali, and of known nitrogen content (Kjeldahl), was used as the standard for the colorimetric procedure. A curve which relates BGG concentration to amounts of precipitated protein was made from standard solutions of BGG included in each batch of unknowns. Blood urea nitrogen was determined in serum by a micro modification of the method of Friedman.[10]

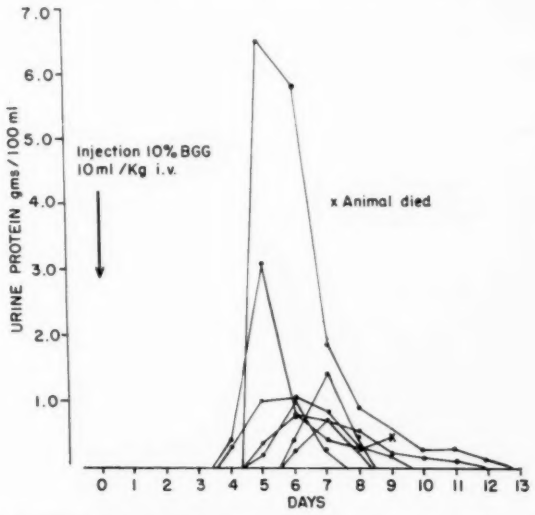


FIG. 2.—Course of the proteinuria which occurred after BGG challenge in animals sensitized by injection of BGG in adjuvant 6 days prior to challenge.

The animals killed for histologic examination were anesthetized with Nembutal, heparinized, and perfused with 2 L. 0.15 M NaCl under pressure by means of a cannula in the abdominal aorta below the origin of the renal arteries. Perfusing fluid escaped from an incision in the inferior vena cava. Tissues were fixed in 10 per cent formalin and stained with hematoxylin-eosin. Tissues regularly examined were kidney, lung, heart, liver, and spleen.

## RESULTS

Previous studies of experimental hypersensitivity have always made use of the pathologic changes in the kidneys to determine the presence of renal disease. When the renal status is followed by tests of serial blood and urine specimens for urea and protein, rather typical changes occur, as illustrated in Fig. 1.

*Occultest Tablets, Ames Company, Inc., Elkhart, Ind.

In this experiment the animal was challenged with BGG, after it had been sensitized to this protein. A proteinuria developed as BGG disappeared from the serum, reaching a level of over 2 Gm. per cent. The blood urea nitrogen (BUN) increased to 50 mg. per cent, and, for 2 days at the height of the nephritis, occult blood was present in the urine.

*Incidence of Nephritis.*—The incidence of nephritis in sensitized animals varied according to the method of sensitization employed. The incidence of proteinuria was greater in sensitized animals than in nonsensitized animals. Proteinuria of 3 or more days' duration occurred in only 7 per cent of nonsensitized animals, while in animals sensitized by adjuvant the incidence was 80 per cent, and in those sensitized by alum-precipitated BGG, 47 per cent (Table I). Minor differences in incidence, which depended on the duration of the period between administration of adjuvant and challenge (2 to 6 days), were not significant.

TABLE I. INCIDENCE AND TIME OF ONSET OF PROTEINURA OF THREE OR MORE DAYS' DURATION AFTER CHALLENGE WITH BOVINE GAMMA GLOBULIN IN SENSITIZED AND NONSENSITIZED RABBITS

| | NUMBER OF ANIMALS | ANIMALS DEVELOPING PROTEINURIA | INCIDENCE OF PROTEINURIA (%) | INTERVAL BETWEEN CHALLENGE AND FIRST PROTEINURIA (DAYS) |
|---|---|---|---|---|
| Nonsensitized | 30 | 2 | 7* | 7 to 10 |
| Sensitized by: | | | | |
| Adjuvant 2 days before challenge | 12 | 10 | 83 | 6 to 8 |
| Adjuvant 4 days before challenge | 17 | 15 | 88 | 4 to 6 |
| Adjuvant 6 days before challenge | 11 | 7 | 64 | 4 to 6 |
| Total adjuvant | 40 | 32 | 80* | |
| Sensitized by: | | | | |
| Alum-precipitated BGG | 17 | 8 | 47* | 4 to 6 |

*Incidence of proteinuria in animals sensitized by alum-precipitated BGG differed significantly from the incidence in those sensitized by BGG in adjuvant ($p = < 0.025$), and from the nonsensitized animals ($p = < 0.005$).

*Severity of Proteinuria and Hematuria.*—The severity and duration of the proteinuria in a group of sensitized animals are given in Fig. 2. Urine protein concentrations reached levels as high as 6.5 Gm. per cent. The duration of proteinuria averaged 5 days, and on occasion lasted as long as 10 days. The ultimate duration of the proteinuria could not be predicted from its initial severity.

Urine from 25 sensitized animals which developed nephritis was tested serially for occult blood. The results were positive in 1 or more specimens in approximately 50 per cent. Gross hematuria was not seen. There was no correlation between hematuria, proteinuria, and azotemia.

*Azotemia.*—Serial determinations of urea nitrogen were made on the blood of 19 sensitized animals which developed nephritis. The BUN rose above 27 mg. per cent in 63 per cent. The elevation was usually transient, lasting 1 or 2 days, and values as high as 60 mg. per cent were not unusual. Animals

with a urine protein concentration not exceeding 1 Gm. per cent did not have azotemia. On the other hand, severe proteinuria was not always accompanied by an elevation of BUN.

*Disappearance of Circulating BGG.*—In the animals receiving BGG after having been sensitized to this protein, the disappearance of BGG from serum was accelerated. The rates of disapperance of BGG in the animals sensitized with the adjuvant and alum preparations are compared with those of the nonsensitized animals in Fig. 3.

In the nonsensitized animals (Curve *A*), the 3 phases of antigen disappearance originally described by Dixon[11] can be distinguished. The first, or equilibration phase, lasted approximately 48 hours, the catabolic phase from day 2 to day 5, and the third, or immune phase, in which rapid removal of antigen occurs by combination with newly formed antibody, continued to day 11. Half-life of BGG, estimated from the catabolic phase of the curve (dashed line extending linear portion of Curve *A*), was 2.35 days.

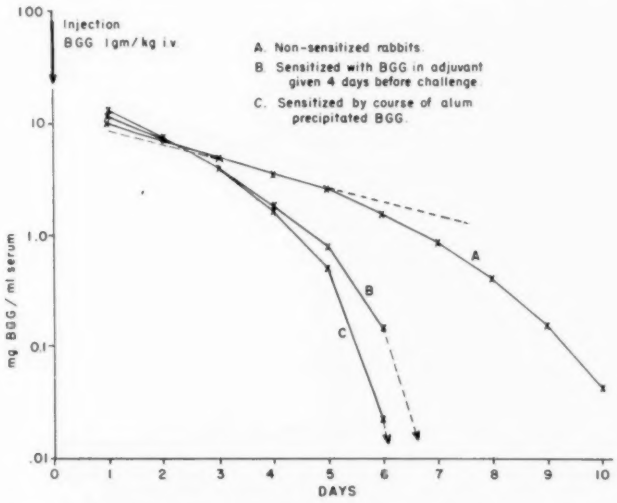


Fig. 3.—Semilogarithmic plot of serum BGG levels in nonsensitized and sensitized rabbits. Curve *A* represents the average of the values for 6 animals; Curve *B*, 11; and Curve *C*, 16 animals.

In the sensitized animals (Curves *B* and *C*), no phase of the curve, solely the result of catabolism, can be distinguished; the pre-existing immunity accelerated the rate of fall from the first. Complete disappearance occurred 5 days earlier than in nonsensitized animals.

The contributions of catabolism and the immue mechanism to BGG disappearance have been calculated for these data by graphing the curves on arithmetic paper (Fig. 4), and determining the differences in area under curves representing actual clearance and catabolic clearance with a half-life of 2.35 days. Disregarding the first 48 hours in which equilibration was taking place, it was calculated that in the nonsensitized animals only 9 per cent of the injected antigen was cleared by the immune mechanism, whereas in the animals

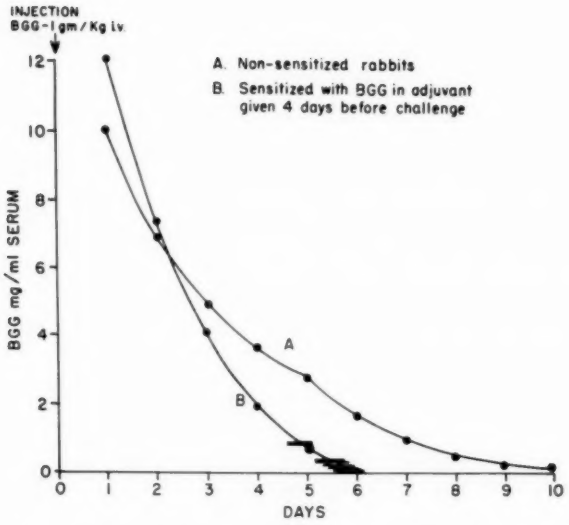


Fig. 4.—Arithmetic plot of serum BGG levels in nonsensitized and adjuvant-sensitized rabbits. Horizontal lines intersecting Curve *B* denote the levels of BGG on the first day of proteinuria in 22 sensitized animals.

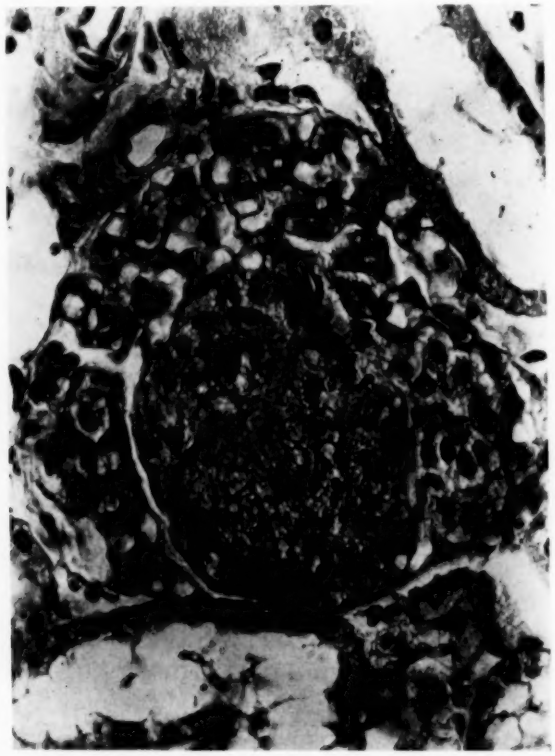

Fig. 5.—Renal glomerulus of a rabbit killed on the first day of proteinuria, 7 days aft intravenous challenge with BGG. Edema and cellular proliferation of glomerular substan has occurred. An encapsulated clot occupies a large portion of the structure. Urine prote 0.5 Gm. per cent, serum BGG level 0.008 mg. per milliliter, BUN 54 mg. per cent at the ti of sacrifice. (×650.)

sensitized by injection of BGG in adjuvant, 30 per cent was cleared by this mechanism. In the animals sensitized by the alum-precipitated preparation, the percentage cleared by the immune mechanism was even greater.

*Relation of Antigen Disappearance to Time of Onset of Proteinuria.*—By the use of proteinuria, rather than microscopic changes in the kidney, to determine the presence of nephritis, the time of onset of nephritis can be easily correlated with the level of circulating antigen. In all cases, the level of circulating BGG was low when proteinuria first appeared. The values are indicated by the point of intersection of the short horizontal lines with Curve *B* in Fig. 4. In 15 of 22 animals studied in this manner, the circulating BGG level was less than 0.15 mg. per milliliter at the time of onset of the nephritis.

*Microscopic Appearance of Kidneys.*—Sixteen animals in the adjuvant-sensitized group were killed for histologic study before or after appearance of proteinuria. The microscopic observations need not be detailed, since they were similar in most respects to those described by others for animals which developed nephritis after injection of bovine serum albumin.

No lesions could be seen in animals killed 1 or 2 days before the expected onset of proteinuria. In those killed on the first day of proteinuria, marked edema and proliferation of the glomeruli were noted, with obliteration of capillary loops and thickening of all structures. Glomerular diameter was increased by one third, on the average. Glomeruli located at the corticomedullary junction frequently showed encapsulated hemorrhage, the result of focal necrosis of capillaries (Fig. 5).[8, 12] Three to 5 days after onset of proteinuria, the

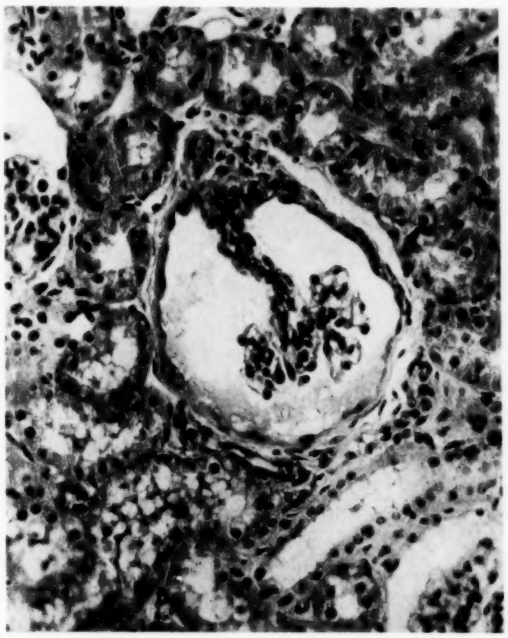

Fig. 6.—Glomerulus of sensitized rabbit killed 24 days after intravenous challenge with BGG. Proteinuria had been present from the fifth to the thirteenth day after challenge. The glomerular capsule is dilated, its epithelium is thickened, and the capsular space contains inspissated protein. The glomerulus has lost much of its substance. (×325.)

glomeruli had shrunk in size, and there was an increase in nuclear density. The capsular space, obliterated at the outset, was now large, and, in some cases, contained inspissated protein. Adhesions between tuft and capsule appeared. Also at this stage, the interstitium of the cortex was infiltrated with plasma cells and lymphocytes in a patchy distribution. In animals killed after disappearance of proteinuria, the glomeruli were normal, except for those in which encapsulated hemorrhage had occurred. These, recognized by their location at the corticomedullary junction, were represented by a large capsule, which appeared either to be empty or to contain a small nubbin of glomerular tissue (Fig. 6).

In other organs, pathologic changes were also observed, but not with the frequency or severity of the nephritis. Most striking was the complete absence of polyarteritis, a lesion seen often after injection of BSA.[1, 3, 8] A mild to moderate myocarditis, endocarditis, and valvulitis were seen in nearly every animal killed after onset of proteinuria. In the arteries of the lung, intimal and endothelial proliferation was seen occasionally, and in 2 animals there were granulomas of the spleen, as described by Germuth.[3]

## DISCUSSION

Numerous previous studies, beginning with those of von Pirquet and Schick[13] at the turn of the century, have given evidence that for the development of the lesions of experimental hypersensitivity, antibody must be available to react with the injected foreign protein. Evidence in this direction consists of the observations that it is not until the immune phase of anigen disappearance that lesions appear,[2, 3] previous sensitization to the protein hastens the appearance of the disease,[4] inhibition of antibody production by prior x-radiation or nitrogen mustard injection prevents the disease,[2] and normal animals which clear antigen slowly because of a poor immune response do not develop lesions.[9] Finally "passive" serum sickness has been produced in rabbits by a constant infusion of homologous antibody after the injection of antigen (BSA).[7]

The mechanism by which a foreign protein and its homologous antibody produce the lesions of experimental hypersensitivity has recently been further elucidated by the demonstration that certain soluble antigen-antibody complexes, formed in antigen excess, have potent immunopathogenic effects. An anaphylactogenic effect of such complexes was first demonstrated by Germuth and McKinnon.[5] Intravenous injection of the supernatants of precipitates of BSA-anti BSA and egg albumin-anti egg albumin, formed in antigen excess, produced an immediate anaphylactic reaction in a high proportion of guinea pigs. In like manner, Trapani and co-workers[14] have shown that complexes of BSA-anti BSA, similarly formed, showed definite activity by the Schultz-Dale technique of in vitro anaphylaxis when guinea pig ileum is used. Injected intradermally, the supernatants also produced skin reactions. From these studies it seems highly probable that the lesions of experimental hypersensitivity are an expression of a protracted anaphylaxis, produced by reaction of the injected antigen with newly formed antibody.

The relatively mild hypersensitivity lesion seen after BGG administration to normal animals might be attributable to 1 of several factors. The protein might (1) be a poor antigen and stimulate production of few antibodies, (2) be cleared from the circulation so rapidly that antibody formation mainly occurs only after antigen has disappeared, or (3) be such that soluble complexes formed with it have only slight anaphylactogenic or cytotoxic effects. From the present study, it would appear that the mild disease is the result of the rapid clearance of BGG from the circulation, with the consequence that complexes with antibody are produced in insufficient numbers for severe disease to result. Although antibodies to BGG appear in the nonsensitized animal 5 days after challenge with BGG, as compared with 10 days for BSA, the more rapid disappearance of BGG by catabolism (half-life 2 to 3 days), as compared with BSA (half-life 4 to 5 days),[15] curtails the number of soluble complexes produced. When clearance of antigen by immune mechanisms is vigorous from the first, as in the sensitized animals of the present study, sufficient complexes are formed to produce a disease comparable in most respects to that which follows BSA injection. The absence of lesions of polyarteritis in the sensitized animals agrees with Germuth's[16] observation that this lesion is dependent on the duration, more than on the intensity, of the immune phase of antigen clearance.

Of interest in the present study is the close correspondence of the onset of the nephritis with the complete or near complete clearance of antigen from the circulation. This correlation has been observed in all studies of these lesions, and has been attributed to the fact that it is only after a delay that antibody is produced. The present results would indicate that this explanation is not complete since, in the sensitized animals, production of antigen-antibody complexes began immediately after challenge with BGG. Failure of disease to be observed immediately is best explained by the lack of cytotoxicity of complexes produced in great antigen excess. In vitro experiments have demonstrated that complexes, formed in antigen excess 60 to 70 times equivalence, are not anaphylactogenic. Maximum effects were found with complexes formed at 6 to 7 times equivalence.[14] These observations would predict that disease would occur only when the antigen level is low.

It is probable that other factors, as yet unknown, influence the cytotoxicity of the soluble complexes. This is suggested by the relatively low incidence of nephritis in the animals sensitized by the alum-precipitated preparation. The disappearance of BGG was as rapid in these animals as in those sensitized with adjuvant. In fact, the animals sensitized with the alum preparation appeared more sensitive in that, despite extensive desensitization, many more succumbed to anaphylaxis on challenge than did those sensitized with the adjuvant. The only explanation at hand for their low incidence of disease is that complexes, formed by antibody produced by this form of stimulation, may not be as uniformly cytotoxic as those formed in response to adjuvant.

SUMMARY

Normal rabbits challenged intravenously with bovine serum gamma globulin develop lesions of experimental hypersensitivity which are mild and usually do not produce a significant proteinuria. Prior sensitization of the animal with bovine serum gamma globulin incorporated in adjuvant markedly potentiates the severity of the disease, and in a high percentage results in a marked proteinuria. Prior sensitization by use of alum-precipitated bovine serum gamma globulin is less effective in potentiating the disease. In 50 to 60 per cent of sensitized animals, hematuria and azotemia accompanied the proteinuria.

Microscopic study of the tissues of animals killed at the height of the proteinuria showed lesions similar to those observed after administration of bovine serum albumin, with the exception that lesions of polyarteritis were absent.

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# THE MINERAL AND WATER COMPOSITION OF HUMAN RED BLOOD CELLS IN DIABETIC ACIDOSIS AND DURING RECOVERY WITH SPECIAL REFERENCE TO CHANGES IN POTASSIUM CONTENT

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DISTURBANCES of the water, potassium, sodium, chloride, and phosphorus content of cells, including red blood cells, in patients with diabetic acidosis have been reported earlier.[1-7] The data in the present report were obtained to clarify the extent of the disturbances in red cell composition.

The effect of early parenteral potassium therapy on the red cell content of potassium was determined and compared with the red cell values of a group of patients with diabetic acidosis who were not given parenteral potassium therapy.[5] The data suggest that early potassium therapy rapidly restores the potassium concentration of plasma and red cells to normal, whereas, without early potassium therapy, reduced concentration of potassium in plasma and red cells may persist for long periods.

*Patients.*—Nine patients with diabetic acidosis were studied. The principles used in guiding fluid therapy were those recommended by Butler.[8] Intravenous saline and lactate were administered initially. Potassium was added as soon as the urine flow was established; usually from 1 to 5 mM. potassium per kilogram body weight was given in the first 24 hours. Glucose was added when the blood glucose fell below 250 mg. per cent or when the urine was free of glucose. Oral fluids, including milk, were usually started before the end of the first 24 hours and routine diabetic diets by the end of the second 24 hours.

*Case Reports.*—The amount of potassium and fluid given in the first 24 hours is indicated for most of the patients. During this time the patients were rehydrated and clinically improved, with the exception of Patient No. 1, who died. The interval between the first and second blood samples will be referred to as the first period (Table I).

*Case 1:* A 15-year-old girl was admitted in coma and with severe dehydration with a history of fatigue, polyuria, polydipsia, and an 11 kilogram weight loss of 1 month's duration. She responded favorably to the administration of large quantities of insulin, fluids containing saline, potassium, lactate and glucose, and whole blood. Fifteen hours after admission she developed shallow respirations and cyanosis. Potassium, which had been included in the intravenous fluid in concentrations up to 50 mM. per liter, was stopped at the sixteenth hour because of the ECG finding of an increased height of the T wave. At the eighteenth hour she rapidly became comatose and died. The development of respiratory paralysis in association with increased height of the T waves was considered to be

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compatible with hyperkalemia. Blood was taken for serum potassium at the sixteenth hour, but, unfortunately, the result was not available until 1 hour after death. A value of 1.6 mM. per liter was found. It was felt that the elevated T waves of the electrocardiogram reflected uncorrected acidosis and obscured the true state of the serum potassium concentration.

*Case 2:* A 50-year-old man who had diabetes for 8 years was admitted in coma with marked hyperpnea and severe dehydration. In the first 24 hours he was given 9,850 ml. fluid, including 1,000 ml. blood, 825 mM. sodium, and 352 mM. potassium, and 2,050 U. insulin. The blood sugar dropped from 600 mg. per cent on admission to 212 mg. per cent at the twenty-second hour, by which time he could be aroused. His clinical condition was normal by the end of the second day. The diabetes was kept well controlled thereafter. His weight on discharge was 50 kilograms.

*Case 3:* A 33-year-old, 60 kilogram, white man with diabetes of 9 years' duration was admitted in semicoma with a history of vomiting, diarrhea, and severe headache of 24 hours' duration. In the first 24 hours, he was given 7,000 ml. fluid containing 600 mM. sodium and 122 mM. potassium; 1,350 U. insulin were required during this time to control the glycosuria and acetonuria. The blood sugar fell from 745 mg. per cent to 272 mg. per cent in the first period. Except for anorexia he was clinically well after the first period.

*Case 4:* A 10-year-old girl with a history of polynuria and polydipsia of 2 weeks' duration, and drowsiness of 24 hours' duration was markedly dehydrated, hyperpneic, and stuporous on admission. In the first 24 hours she was given 350 ml. blood and 4,000 ml. fluid containing 290 mM. sodium and 150 mM. potassium, and 970 U. insulin. The blood sugar fell from 352 mg. per cent to 152 mg. per cent by the end of the first period, after which time her condition was well regulated. Her admission weight was 22.2 kilograms, and her discharge weight was 24.6 kilograms.

*Case 5:* A 26-year-old woman, diabetic for 21 years, developed vomiting and abdominal pain 2 days before admission. She was moderately dehydrated and confused on admission and had marked Kussmaul respirations. In the first 24 hours she was given 6,980 ml. fluid containing 300 mM. sodium and 76 mM. potassium. Her sensorium was normal by the ninth hour. The blood sugar fell from 776 mg. per cent on admission to 267 mg. per cent at the tenth hour. The urine was free of acetone and glucose by the twelfth hour; a total of 810 U. insulin was given during the first period. The discharge weight was 48 kilograms.

*Case 6:* A 66-year-old woman, diabetic for many years, started vomiting 36 hours before admission and appeared to be somewhat lethargic and moderately dehydrated on admission. During the first 24 hours 9,850 ml. fluid containing 240 mM. sodium and 209 mM. potassium, and 120 U. insulin were administered. The blood sugar dropped from 812 mg. per cent on admission to 374 mg. per cent by the seventh hour. Oral fluids were started by the third hour after which time she remained clinically normal. The diabetes was well controlled after the first period. The discharge weight was 52 kilograms.

*Case 7:* A 26-year-old woman, diabetic for 14 years, omitted insulin for 24 hours and started vomiting 8 hours before admission. She was moderately dehydrated and lethargic on admission. During the first 24 hours she was given 9,790 ml. fluid containing 555 mM. sodium and 120 mM. potassium, and 650 U. insulin. The blood sugar fell from 644 mg. per cent to 217 mg. per cent by the end of the first period. Her clinical condition was normal 6 hours after admission. Her weight was 47.8 kilograms on admission and 51 kilograms on discharge.

*Case 8:* A 15-year-old girl, diabetic for 2 years, was admitted with a history of vomiting and dizziness of 2 hours' duration. On admission she was moderately lethargic with moderate Kussmaul respirations and mild dehydration. She was judged to be clinically normal after 8 hours of therapy, during which time she received 200 U. insulin, 1,600 ml. fluid, containing 150 mM. sodium and 78 mM. potassium. After the eighth hour she was given milk ad libitum. The intake of potassium in the first period was 160 mM. The urine was free of acetone at the tenth hour and free of glucose by the eighteenth hour. Her discharge weight was 45.4 kilograms.

*Case 9:* A 17-year-old girl, with diabetes since birth, omitted insulin 24 hours before entry and started vomiting 12 hours later. On admission she was moderately dehydrated and hyperpneic, but was mentally alert. In the first 24 hours she was given 10,055 ml. fluid containing 450 mM. sodium and 128 mM. potassium. The diabetes was kept well controlled and she ate normally after the first period. The blood sugar dropped from 625 mg. per cent on admission to 153 mg. per cent by the end of the first period. Her discharge weight was 66.8 kilograms.

## METHODS

The direct method was used for red cell analysis.[9] Blood samples of 10 to 20 ml. were drawn, without the use of a tourniquet, from the antecubital vein into unoiled silicone-coated syringes which contained a trace of heparin. The blood was kept in the stoppered syringe at room temperature with negligible loss of $CO_2$. When $CO_2$ or pH was to be determined, a separate sample of blood was kept iced under mineral oil.

A well-mixed aliquot was transferred immediately to a rubber-stoppered Wintrobe tube for hematocrit determination. An appropriate amount of blood, as determined by the hematocrit or whole blood hemoglobin, was then transferred to a silicone-coated, rubber-capped, Butler-Cushman-type constricted centrifuge tube, so that the buffy layer would be in the constricted portion of the tube. This permitted the complete removal of supernatant plasma and buffy layer. The specimens were spun at 4,100 r.p.m. (mean radius of gyration of 15 cm.) for 45 minutes at 20° C. After centrifugation the supernatant plasma and buffy layer were removed. The entire red cell sample was pipetted into a tared ground-glass stoppered weighing bottle and weighed. The sample was then diluted with an accurately measured volume of distilled water, about 4 ml. water per gram of red cells.

Sodium and potassium were determined by flame photometry with a Barclay flame photometer with an internal lithium standard.[10]

Total phosphorus was determined by the method of Fiske and Subbarow,[11] chloride by a modification of the Volhard method, silver nitrate and nitric acid being added separately as recommended by Wilson and Ball,[12, 13] magnesium by a modification of the method of Briggs and Denis,[14, 15] and $CO_2$ content by the method of Van Slyke and Neill.[16] The water content was determined by drying specimens for 24 hours in an oven at 105° C.

*Calculations and Methods of Presenting Red Cell Data.*—The analytic values of cell constituents as grams or millimoles per kilogram of cells were converted to quantities per kilogram cell solids by dividing the former values by the fraction of solids per kilogram cells. No correction was made for trapped plasma. The formula that can be used to convert quantities per kilogram cell solids to quantities per kilogram cells, or cell water, is presented elsewhere.[9]

The conventional methods of presenting red cell electrolyte data as quantities per weight or volume of cells or as quantities per weight or volume of cell water may be misleading for description of change in water and mineral content. For example, the loss of 53 Gm. water from one kilogram of red cells would produce an increase in potassium of 5.6 per cent if the potassium referred to whole cells; if referred to red cell water, the increase would be 9.0 per cent. However, if potassium content referred to cell solids, no change would result. The use of cell solids as a reference will avoid apparent changes in electrolyte content caused by changes in water content alone.

## RESULTS

Data obtained from the analysis of red cells and plasma of 9 patients studied after admission for diabetic acidosis are listed in Table I. Patients Nos. 1 to 4 were clinically severely ill and Patients Nos. 5 to 9 were mildly to moderately ill. On admission the red cell water content was normal in all but one mildly ill patient (No. 9), in whom an elevated value was found.

TABLE

| PATIENT (NO.) | AGE (YEARS) | SEX | TIME AFTER ADMISSION (DAYS) | RED CELLS (PER KG. SOLIDS) H₂O (GM) | NA (MM.) | K (MM.) | CL (MM.) |
|---|---|---|---|---|---|---|---|
| 1 | 15 | F | 2/24 | 1,861 | 28.0 | 195 | 226 |
| 2 | 50 | M | 0 | 1,860 | 23.5 | 197 | 199 |
| | | | 17/24 | 1,797 | 27.1 | 210 | 151 |
| | | | 15 | 2,047 | 49.7 | 266 | 173 |
| 3 | 33 | M | 0 | 1,933 | 42.5 | 209 | 170 |
| | | | 4/24 | 1,874 | 41.7 | 204 | 138 |
| | | | 7/24 | — | — | — | — |
| | | | 8 | 1,933 | 51.3 | 240 | 138 |
| | | | 100 | 2,054 | 42.8 | 260 | — |
| 4 | 10 | F | 0 | 1,880 | 29.9 | 204 | 204 |
| | | | 16/24 | 1,686 | 36.3 | 213 | 107 |
| | | | 3 | 1,838 | 37.5 | 227 | 136 |
| | | | 4 | 1,997 | 50.0 | 267 | 149 |
| | | | 8 | 1,978 | 44.6 | 258 | 123 |
| | | | 63 | 1,978 | 35.7 | 259 | 155 |
| 5 | 26 | F | 0 | 1,950 | 37.2 | 244 | 171 |
| | | | 1 | 1,846 | 36.1 | 249 | 109 |
| | | | 4 | 1,941 | 51.4 | 266 | — |
| | | | 6 | 1,990 | 44.3 | 270 | 141 |
| | | | 16 | 2,006 | 50.8 | 276 | — |
| 6 | 66 | F | 0 | 2,006 | 37.6 | 246 | 154 |
| | | | 6/24 | 1,909 | 33.4 | 250 | 122 |
| | | | 1 | 2,032 | 31.2 | 255 | 123 |
| | | | 3 | 1,918 | 41.7 | 263 | 143 |
| | | | 7 | 1,919 | 44.7 | 264 | 162 |
| 7 | 26 | F | 0 | 1,990 | 25.7 | 246 | 200 |
| | | | 1 | 1,934 | 31.4 | 253 | 147 |
| | | | 2 | 1,936 | 34.7 | 269 | 133 |
| | | | 9 | 1,933 | 48.2 | 281 | — |
| | | | 73 | 2,031 | — | 284 | — |
| 8 | 15 | F | 0 | 2,001 | 35.7 | 260 | 155 |
| | | | 1 | 1,930 | 41.3 | 265 | 120 |
| | | | 4 | 2,003 | 39.3 | 289 | 156 |
| 9 | 17 | F | 0 | 2,168 | 25.3 | 255 | 187 |
| | | | 18/24 | 2,058 | 34.8 | 286 | 119 |
| | | | 2 | 1,981 | 39.3 | 283 | 125 |
| | | | 4 | 2,101 | 37.3 | 283 | 155 |
| | | | 210 | 2,009 | 35.5 | 291 | 138 |
| | | | 22 normal adults[s] | 1,948 | 39.5 | 272 | 153 |
| | | | S. D. | 55 | 3.0 | 9.5 | 9 |
| | | | 6 diabetic patients with vascular and renal complications of diabetes but no acidosis. | 1,982 | 39.4 | 263 | 151 |
| | | | Low | 1,926 | 31.9 | 258 | 130 |
| | | | High | 2,051 | 47.8 | 289 | 164 |

During the first 6 to 24 hours of treatment there was a reduction in the red cell water content in all patients, falling below the normal range in 2 severely ill patients. A return to normal occurred during convalescence (Fig. 1, *A*).

On admission, the potassium content of red cells was below normal in 7 patients; in 2 mildly ill patients (Nos. 8 and 9) it was in the lower range

| | | PLASMA | | | | | | |
|---|---|---|---|---|---|---|---|---|
| P (MM.) | MG (MM.) | $H_2O$ (GM./KG.) | NA (MM./KG.) | K (MM./KG.) | CL (MM./KG.) | $CO_2$ (MM./L.) | P (MM./L.) | pH |
| 26.8 | — | 908 | 135.7 | 3.1 | 98.5 | 3.7 | 1.6 | 6.90 |
| 38.3 | 10.9 | 901 | 132.1 | 3.4 | 84.0 | 7.4 | 2.2 | 7.05 |
| 40.8 | 10.0 | 931 | 138.4 | 2.7 | 96.7 | 22.9 | 0.9 | 7.35 |
| 56.4 | 10.7 | 919 | 138.0 | 4.2 | 97.8 | — | — | — |
| 46.3 | — | 901 | 128.9 | 6.5 | 84.0 | 3.7 | — | 7.08 |
| 46.3 | — | 926 | 134.0 | 3.4 | 100.0 | 9.4 | — | — |
| — | — | — | — | — | — | 21.3 | — | 7.45 |
| 54.0 | — | 915 | 136.7 | 4.6 | 102.5 | — | — | — |
| 62.0 | — | — | — | 4.5 | — | — | — | — |
| 37.5 | — | 898 | 131.2 | 3.2 | 104.5 | 3.6 | 1.0 | 7.00 |
| 41.1 | — | 936 | 142.6 | 1.9 | 101.0 | 20.1 | 0.3 | 7.35 |
| 44.3 | — | 945 | 132.4 | 4.3 | 98.6 | 25.4 | 0.9 | 7.47 |
| 67.4 | — | 930 | — | — | — | — | — | — |
| 58.6 | — | 929 | 138.4 | 3.9 | 104.9 | — | — | — |
| 58.6 | — | 916 | 138.0 | 5.0 | 103.5 | — | — | — |
| 47.5 | — | 895 | 127.6 | 5.4 | 88.9 | 4.9 | 2.4 | 7.15 |
| 40.3 | — | 920 | 143.0 | 3.4 | 104.5 | 28.7 | 0.2 | 7.41 |
| 62.6 | — | 913 | 138.4 | 5.5 | 100.6 | 28.6 | 1.0 | — |
| — | — | 912 | — | — | — | — | — | — |
| 61.0 | — | 914 | 137.9 | 3.9 | 96.3 | — | — | — |
| 44.5 | — | 900 | 130.6 | 4.8 | 90.6 | 11.7 | 1.5 | 7.21 |
| 45.1 | — | 920 | 133.1 | 3.9 | 93.5 | 26.8 | 0.5 | 7.45 |
| 54.6 | — | 927 | 127.7 | 4.8 | 90.8 | 27.7 | 0.5 | 7.41 |
| 63.6 | — | 913 | 129.3 | 3.8 | 96.4 | 29.9 | 1.0 | 7.45 |
| 58.1 | — | 917 | 129.5 | 4.1 | 96.4 | — | 0.9 | — |
| 45.4 | — | 916 | 132.8 | 3.9 | 103.5 | 3.9 | — | 7.12 |
| 50.0 | — | 933 | 134.9 | 3.6 | 104.2 | — | — | — |
| 56.1 | — | 928 | 138.6 | 4.7 | 105.0 | — | — | — |
| 59.9 | — | 915 | 137.6 | 4.2 | 104.6 | — | — | — |
| 61.8 | — | — | — | 4.6 | — | — | — | — |
| 57.3 | 12.5 | 910 | 134.2 | 4.8 | 99.5 | 12.0 | 1.4 | 7.20 |
| 59.5 | — | 918 | 138.3 | 3.5 | 101.5 | 28.5 | 1.3 | 7.32 |
| 67.9 | 12.1 | 918 | 139.0 | 4.4 | 102.5 | — | 1.3 | — |
| 52.0 | — | 902 | 126.0 | 5.9 | 93.6 | 7.9 | 2.2 | 7.15 |
| 53.2 | — | 939 | — | 3.8 | — | 26.8 | 0.6 | 7.40 |
| 54.1 | — | 929 | — | 4.9 | — | 31.5 | — | — |
| 62.9 | — | 920 | 142.5 | 4.9 | 102.6 | — | — | — |
| 81.6 | — | — | — | 4.2 | — | — | — | — |
| 58.0 | | 914 | 141.3 | 4.0 | 101.2 | 28.0 | | 7.43 |
| 7.2 | | 3.7 | 1.1 | 0.2 | 2.5 | 2.0 | | 0.03 |
| 62.5 | | 907 | 137.7 | 4.7 | 100.0 | — | | — |
| 59.7 | | 900 | 131.0 | 3.4 | 93.0 | | | |
| 68.6 | | 915 | 148.0 | 5.8 | 105.0 | | | |

rmal (Table I). In every patient there was a rise in the potassium content th therapy (Table I, Fig. 1, *B*). Red cells in 4 patients (Nos. 4-7) contained normal amount of potassium after 3 days of therapy, but in 1 severely ill tient (No. 3) the red cell contained less potassium than normal for more than days.

The total body retention of potassium in cells was measured in 5 patients (Table II). A positive balance of potassium in red blood cells and in the average body cell was seen in all cases.

The sodium content of red cells on admission was below normal in 5 patients, and within normal limits in the other 4 (Table I, Fig. 1, *C*). During

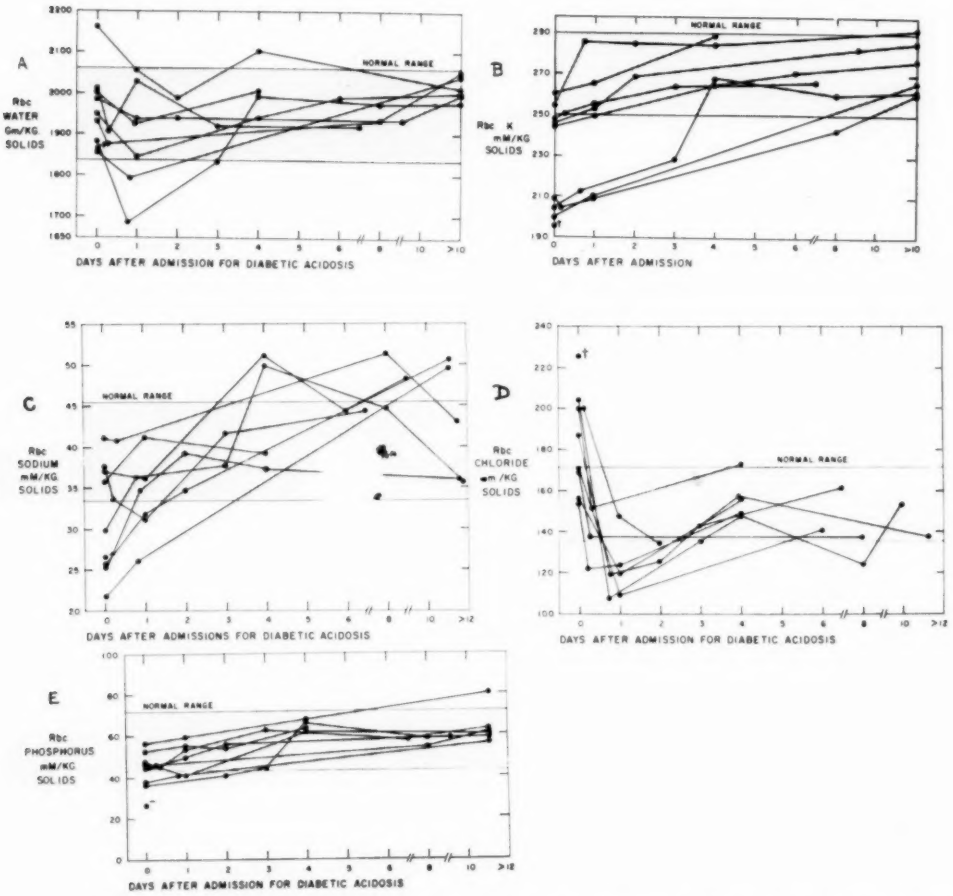


Fig. 1.—The electrolyte and water content of red cells in diabetic acidosis and during recovery: *A*, red cell water content; *B*, red cell potassium content; *C*, red cell sodium content; *D*, red cell chloride content; and *E*, red cell phosphorus content.

recovery, there was an increase in red cell sodium content in all patients, reaching values above normal limits in 5 patients. The red cell magnesium content was measured in 2 patients; the observed change did not exceed analytic variability (Table I).

The chloride content of red cells was elevated in 5 patients on admission. A fall, often to an abnormally low value, was observed in 8 patients in the first 24 hours. This was followed by a rise to normal during convalescence (Fig. 1, *D*).

The red cell phosphorus was reduced below the normal range in 3 severely ill patients; the reduction in Patient No. 1, who died, was over 50 per cent of the normal phosphorus content of red cells. In all patients a gradual rise which paralleled the red cell potassium changes was observed during recovery (Table I, Fig. 1, *E*).

The electrolyte composition of plasma on admission was similar to that previously reported in patients with diabetic acidosis.[1-7] There was no simple relationship between the plasma potassium concentration and the red cell potassium content. Reduced plasma potassium concentrations were present in 3 patients (Nos. 1, 2, and 4) on admission, despite the presence of severe acidosis.

The water and electrolyte composition of red cells in 6 additional patients with diabetes mellitus without acidosis was normal (Table I). These data further indicate that diabetes mellitus per se does not result in the changes in the composition of red cells found in diabetic acidosis.

TABLE II. CONCURRENT RETENTION OF POTASSIUM IN RED CELLS AND TOTAL BODY BALANCE OF POTASSIUM OF CELLS OF 5 PATIENTS DURING TREATMENT FOR DIABETIC ACIDOSIS

| PATIENT (NO.) | TIME STUDIED AFTER ADMISSION FOR DIABETIC ACIDOSIS (DAYS) | TOTAL BODY CELL* BALANCE OF POTASSIUM (MM. K/KG. BODY WT.) | RED CELL BALANCE OF POTASSIUM (MM./KG. R.B.C. SOLIDS) |
|---|---|---|---|
| 2 | 0-1 | 3.8 | 13 |
| 5 | 0-4 | 4.1 | 22 |
| | 4-16 | 1.9 | 10 |
| 6 | 0-3 | 3.2 | 17 |
| 7 | 0-2 | 3.5 | 23 |
| 8 | 0-1 | 2.7 | 5 |

*The total body balance of potassium was corrected for the change in the extracellular potassium content, and in Patients Nos. 2, 5, and 7 for a change in the total body nitrogen balance, using a K:N ratio of 2.8.[25]

## DISCUSSION

*Red Cell Water Content.*—The red cell water content tends to be variable in diabetic acidosis. In this condition factors are present which exert opposite changes in red cell water content. An increase in red cell water content is effected by the chloride shift accompanying acidosis, and by a reduction in the extracellular sodium concentration.[17, 18] A decrease in red cell water content is produced by water deprivation[19] and by loss of intracellular solutes (potassium, sodium, and phosporus[20]). In man (and in the anthropoid apes) changes in plasma glucose concentration are accompanied by equal changes in the glucose concentration of red cell water, and, therefore, do not affect red cell water content directly.[21] During diabetic acidosis, the red cells showed a correlation of water content with potassium, suggesting that the latter was an important factor (Fig. 2). In the first 24 hours of treatment, a decrease in red cell water content in all patients was related to correction of acidosis. An increase in pH is associated with a loss of red cell chloride content (Fig. 3).

*Red Cell Potassium Content: Possible Relation to Total Body Cell Potassium Status in Diabetic Acidosis.*—Previous balance studies indicate that patients recovering from diabetic acidosis retain from 2 to 14 mM. potassium in excess

of nitrogen per kilogram body weight.[1-3] This is about 5 to 30 per cent of the normal total body exchangeable potassium content.[22-24]

Data presented in this report show that red cells from patients with diabetic acidosis also contain 5 to 30 per cent less potassium than normal, the deficit being greater in the severely ill patients than in the mild to moderately ill patients. Repletion of the red cell potassium content to normal occurred within 2 to 3 days in the mildly ill patients but required a longer period in the severely ill patients. In 3 patients (Nos. 2, 4, and 5) the red cell potassium content was still markedly reduced 2 to 3 days after admission when, clinically, they were greatly improved. These data suggest that cell deficit of potassium may not be related to the acute clinical symptoms of diabetic acidosis. The absence

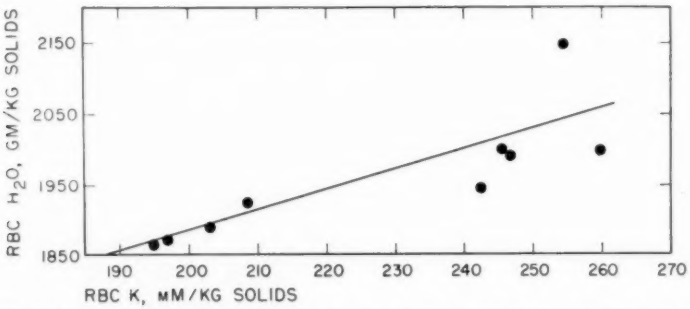


Fig. 2.—The relationship of the water and potassium content of red cells in diabetic acidosis (admission findings); r = +0.82, P = 0.01.

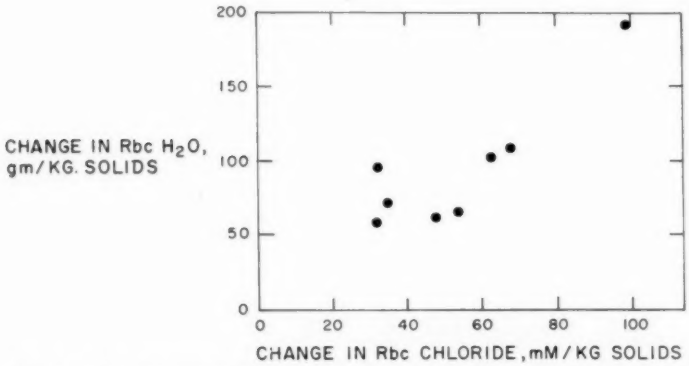


Fig. 3.—The relationship of the red blood chloride content to the red cell water content in the first 24 hours of treatment of diabetic acidosis; r = 0.84, P = <0.01.

of clinical symptoms in the presence of marked potassium deficit of cells has been noted elsewhere.[26] The balance data of Nabarro[3] and of our Case 5,* indicate that total body retention of potassium in excess of nitrogen is gradual and continues for several days during recovery from severe diabetic acidosis. The balance data presented in Table II can be interpreted to indicate that the per cent increase in potassium of all body cells may be quite similar to the per cent increase in potassium content of red cells.[25]

*Complete balance data of Patient No. 5 appear elsewhere.[25]

Nichols and Nichols[5] have reported the chemical composition of red cells of a group of patients with diabetic acidosis not treated with parenteral potassium. The potassium concentration in red cell water of their patients showed an increase during recovery in only 5 of 9 patients and in some patients was lower on discharge than on admission. In several of the patients plasma potassium concentrations as low as 2.5 mM. per liter were seen 2 to 7 days after admission (Fig. 4); however, only normal plasma potassium values were seen after the first treatment day in the patients in the present series who were given potassium parenterally (Fig. 4).

The concentration of potassium in plasma often is not a dependable index of the total body cell potassium status during diabetic acidosis, since high values are often found in patients who have marked cell potassium depletion.[3] This may in large part be due to the effect of acidosis on the cell to plasma potassium concentration ratio, as reported by Burnell and co-workers.[27] However, after dehydration, ketoacidosis, and renal dysfunction have been corrected,

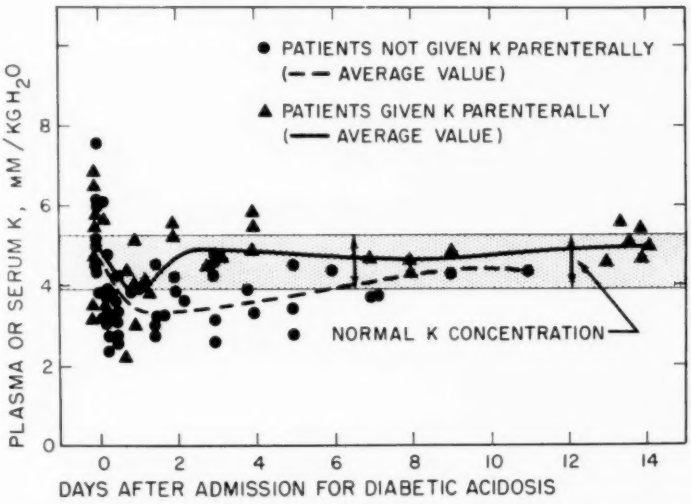


Fig. 4.—The plasma or serum concentration of potassium in patients treated with parenteral potassium (triangles, present series) compared to the values found in patients not given parenteral potassium (circles, patients of Nichols and Nichols[5]).

low potassium concentrations are frequently seen. The finding of Holler[28] and others, that the muscle paralyses which develop during the early treatment of diabetic acidosis are accompanied by marked hypokalemia and respond promptly to intravenous potassium, indicates the importance of avoiding hypokalemia. Our Case 1 appears to illustrate this point.

Nabarro's data of the calculated total intracellular potassium status during recovery from diabetic acidosis show a much greater retention when potassium is administered early.[3] The same finding is suggested by the data of Seldin and Tarail,[7] and Danowski.[6] It is not clear why the early parenteral*

*Oral potassium therapy would be expected to accomplish the same effect, but in the first 12 hours of treatment patients with diabetic acidosis frequently vomit, and a large oral intake of potassium may be contraindicated.

administration of potassium results in a more rapid accumulation of potassium after the first 24 hours of treatment, because the only source of potassium after this time is usually food. Possibly, the patients given early parenteral potassium have a better appetite.

*Red Cell Phosphorus.*—The balance data of Atchley and Loeb[1] and of Butler and co-workers[2] showed that mildly acidotic, insulin-deprived diabetic subjects lost little or no phosphorus in excess of nitrogen and calcium. The balance data of Nabarro and co-workers[3] obtained in patients with severe diabetic acidosis showed that large amounts of phosphorus were lost in excess of

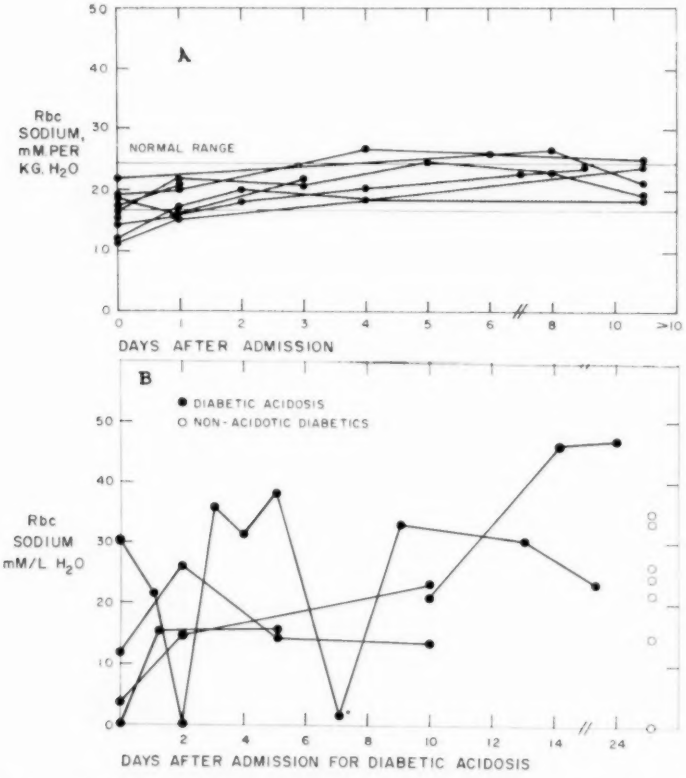


Fig. 5.—The concentration of sodium in red cells as determined by (*A*) the direct method (present report), and (*B*) as determined by the indirect method (patients of Danowski, Hold, and Peters[34]).

nitrogen.* The latter data support the suggestion of Guest[29] that acidosis results in a decomposition of organic phosphates in many body cells, in addition to red cells, However, the per cent loss of phosphorus from red cells apparently is greater than the per cent loss of phosphorus from the average body cell.[3]

*Red Cell Chloride.*—It has been shown by Van Slyke and associates[17] that the chloride concentration of red cells is dependent on the extracellular chloride

*Calcium balances were not obtained.

and hydrogen ion concentrations and on the cellular and extracellular concentrations of nondiffusable anions. The largest loss of chloride from red cells after treatment in our patients was 97 mM. per kilogram solids (Patient No. 4). Since a 70 kilogram adult may have 1 kilogram of red cell solids,[30] the chloride shift in red cell should be taken into consideration if chloride balances are used to derive fluid space measurements.

*Red Cell Sodium.*—In potassium deficiency not associated with acidosis an elevation of the sodium content of red cells as well as muscle cells is usually observed.[31-32] When acidosis is superimposed upon potassium deficiency, the usual elevation in cell sodium in muscle may not be pronounced or may be absent.[33] Knowles and Guest[34] have reported normal muscle sodium values in potassium deficient diabetic acidotic rats. As reported originally by Danowski and co-workers,[35] the sodium and potassium concentration of red cells do not change reciprocally in patients with diabetic acidosis, both ions usually being lost.

We are unable to explain the elevation in red cell sodium content which occurred in 5 patients during convalescence. In 4 of the 5, the cell potassium content had already returned to normal. However, none of the changes in red cell sodium concentration of our patients were as labile as those reported by others who used the indirect method for analyzing red cells (Fig. 5). As discussed elsewhere, greater analytic variability is to be expected when the indirect, rather than the direct, method of analyzing red cells is used.[9]

The osmotic adjustments with plasma which accompany the red cell water and electrolyte content changes have been presented in detail elsewhere.[35]

## SUMMARY

Red cell water and electrolyte composition was determined in 9 patients with diabetic acidosis. Using cell solids as a reference, the following were observed: (1) a reduced potassium content, the reduction being roughly correlated with the severity of illness, (2) reduced or low normal sodium and phosphorus values, and (3) a gradual increase in the potassium, sodium, and phosphorus content in 3 to 8 or more days, with treatment which included parenteral potassium. In one half of the patients there was a transient elevation of the red cell sodium content during convalescence. The water content of red cells on admission ranged from low normal to high, and was correlated directly with the red cell potassium content. During the first day of treatment the water content fell in all instances with a simultaneous reduction of the red cell chloride content.

The red cell and plasma potassium concentrations of a group of patients with diabetic acidosis treated with parenteral potassium were compared to another group of patients reported upon elsewhere,[5] who were not treated with parenteral potassium. Reduced plasma potassium concentrations after the first treatment day were found only in the group not treated with parenteral potassium. A gradual restoration of the red cell potassium concentration to normal

was observed in all patients who received early parenteral potassium but the red cells of some of the patients who did not receive parenteral potassium became more depleted of potassium during the early treatment for diabetic acidosis.

Evidence of a correlation between the degree of red cell potassium depletion and the magnitude of total body cell potassium deficiency is presented.

We wish to acknowledge the support and advice of Dr. A. M. Butler, Massachusetts General Hospital, and Drs. R. Berliner and E. Cotlove, National Institutes of Health.

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# DEMONSTRATION OF HEPATIC MAXIMUM REMOVAL CAPACITY ($L_m$) FOR GALACTOSE IN HUMANS

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WHEN substances are transferred across living membranes by processes which use energy derived from cellular metabolism, the transfer is known as "active transport."[1] One important characteristic of such transport is that when increasing loads of the substance transported are presented, a fixed maximal transfer rate is eventually reached (saturation), beyond which transfer cannot be increased no matter how great the load. For the kidney, transport maxima ($T_m$'s) for substances which are reabsorbed or secreted by the renal tubule have been demonstrated and carefully characterized.[2]

Several authors have shown that the liver has a maximum removal capacity for sulfobromophthalein (BSP) and have used the term "$L_m$" to represent it (and presumbably other hepatic transport maxima, as well).[3-5] Recently, the theory for a noncatheterization clearance method for estimating hepatic blood flow was presented, which is based upon the use of a test substance for which the liver has such an $L_m$.[6] However, direct evidence for the existence of hepatic $L_m$'s for substances other than BSP which are removed from the blood, or secreted into the blood or bile by the liver, has been lacking. The purpose of the present report is to provide such direct evidence of an $L_m$ in the normal human liver for galactose. The use of galactose to demonstrate an $L_m$ (in this instance, a removal $L_m$) was suggested by the observed behavior of this substance after single intravenous injection.[6] Constant infusion to concentration equilibrium was used to minimize error caused by the rates of diffusion into, and mixing within the ultimate volume of distribution. Galactose titration was used to demonstrate the $L_m$ in a manner similar to the classical use of glucose titration to demonstrate the glucose reabsorptive $T_m$ of the renal tubule.[7]

## MATERIALS AND METHODS

Fifteen men, between 22 and 62 years of age, convalescent from a variety of nonhepatic disorders, were the subjects of this study. Physical examination and a battery of liver tests were negative for hepatic abnormalities in every subject. All studies were done with the subject in a fasting condition and sedated with promazine hydrochloride 50 mg. intramuscularly.

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After voiding and discarding the urine, the subject drank 1 to 1½ L. of water to insure an adequate output, so that subsequent urine collection did not require urethral catheterization and its attendant hazards.[8] From time to time he drank additional water to maintain urine output. Urine was collected after a timed interval of approximately 30 minutes, the volume recorded, and an aliquot used to measure nongalactose-reducing substances, the rate of excretion of which varied from 0.5 to 3.5 mg. per minute (average 1.1 mg. per minute). It was assumed that the excretion of these substances was constant throughout the experimental period. The control rate of excretion was subtracted from the excretion rate of total reducing substances in the subsequent galactose-containing urine samples to obtain galactose excretion rates during the experimental periods.

Constant infusion of galactose at a predetermined rate was accomplished by means of a motor-driven syringe pump* which was known to deliver either 1.69 or 2.54 ml. per minute depending upon the gear ratio used. The 50 ml. pump syringe was refilled during the infusions from a reservoir connected to it by means of a threeway stopcock. The maximum time of interruption of an infusion in order to refill the syringe was 105 seconds, but interruptions averaged only 33 seconds. Priming doses were injected through the stopcock. The syringe was connected by polyethylene tubing of PE 240 gauge to an indwelling 19 gauge needle inserted into an antecubital vein of the left arm.

Peripheral blood samples, each 3 to 4 ml. in volume, were obtained by means of an indwelling 18 gauge Cournand needle inserted into an antecubital vein of the right arm. Samples from the hepatic vein were obtained by means of a F7 Nylon cardiac catheter which had been introduced into the left cephalic vein and advanced into a right hepatic vein under fluoroscopic guidance. Each blood sample was immediately transferred to a 50 ml. flask containing dried heparin as the anticoagulant and thoroughly mixed.

In 1 subject, a single infusion of galactose was carried out for 79 minutes to demonstrate that peripheral concentration equilibrium was attainable and maintainable. In each of the next 12 subjects, 2 rates of infusion (galactose titration) were carried out as follows: a 10 ml. control sample of galactose-free peripheral blood was obtained. The first (and slower) infusion was then begun and 30 minutes allowed for equilibrium to be reached, at which time the first urine sample was collected. Three to 7 peripheral blood samples were then collected at approximately 5 minute intervals, after which the second urine sample was collected. A second and faster infusion was then started. This was accomplished either by changing the gear ratio of the pump and using the original infusate, or by maintaining the original gear ratio and changing to an infusate of greater concentration. If the second rate of infusion was much greater than the first, a priming injection of 50 per cent galactose was given. After another 30 minutes to permit equilibration, urine and peripheral blood samples were obtained as before. All sample collections and interruptions of the pump for refilling or change of infusion were accurately timed by a stopwatch, with the start of the first infusion as reference time. It will be noted that the second and fourth urine samples were the ones collected during the equilibrium period. Finally, in each one of 2 additional subjects, hepatic vein catheterization was performed in conjunction with a single galactose infusion. In these, blood from peripheral and hepatic veins was sampled frequently throughout the entire infusion period.

The galactose solutions that were infused were prepared to approximate concentrations by the addition of 0.9 N NaCl to 50 per cent galactose solution (w/v) for intravenous injection (Pfanstiehl). A sample of each infusion was later analyzed for exact concentration. This permitted calculation of exact infusion rate from the known volume delivery rate of the calibrated infusion pump. Infusions and urine samples were analyzed for galactose by the Nelson-Somogyi hexose method.[9] Galactose in whole blood also was analyzed by this method after glucose in blood had first been eliminated by incubation with highly purified glucose oxidase.[10] Repeated analyses done by this technique are accurate to within 5 per cent in our experience. Regardless of blood concentration, 5 per cent of the galactose was

*Aminco Motor-Driven Compensator, American Instrument Co., Silver Springs, Md.

lost because of the glucose oxidase,[19] and was corrected for in the final calculation of galactose concentration. The small amount of nonhexose-reducing substance present in the blood was measured after treatment of the control-blood sample with glucose oxidase. This amount was subtracted from all subsequent galactose-containing samples. A standard solution of galactose, added to the control blood, and analyzed with, and without, glucose oxidase treatment, served to demonstrate that neither enzyme inhibition, nor unusual enzyme activity against galactose, occurred in the samples tested.

Calculations from the results obtained during the equilibrium periods were: (1) $R = I - U$, (2) $Cl_H = \frac{R}{P_{eq}}$, and (3) $Cl_r = \frac{U}{P_{eq}}$, where R is the removal rate of galactose within the body (mg. per minute), I is the rate of infusion (8 mg. per minute), U is the averaged urine excretion of galactose (8 mg. per minute), $Cl_H$ is the "hepatic" clearance of galactose (milliliter per minute), $P_{eq}$ is the averaged peripheral blood concentration (milligram per 100 ml.), and $Cl_r$ is the renal clearance of galactose (milliliter per minute). Only the urine samples collected during the equilibrium periods were used in the caculations.

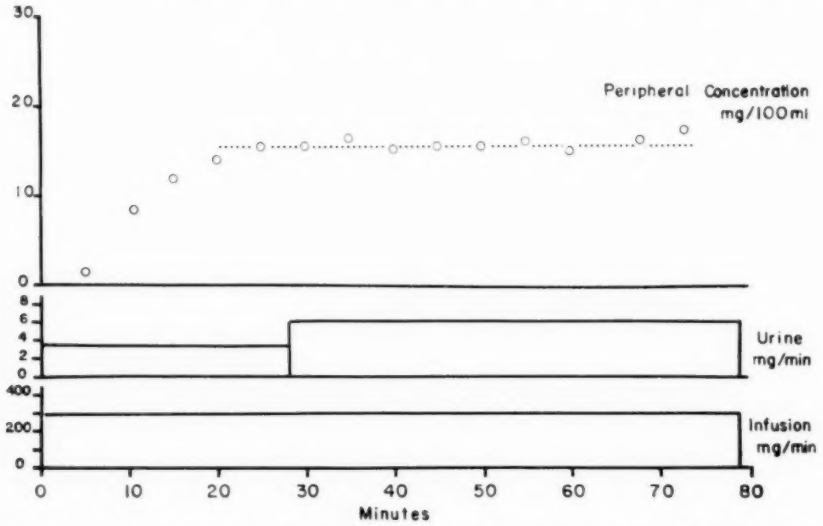


Fig. 1.—Results of infusion of galactose at the constant rate of 292 mg. per minute for 79 minutes. The dotted line represents the arithmetic mean of the 10 values for P obtained during the concentration equilibrium, and is referred to as $P_{eq}$ in the text.

## RESULTS

Fig. 1 illustrates that concentration equilibrium can be attained and maintained for a considerable period. In this subject, a constant infusion of galactose at the rate of 292 mg. per minute was given for 79 minutes. A priming injection of galactose was not given. The peripheral blood concentration of galactose (P) rose steadily, until a level of 15.65 mg. per 100 ml. was reached after 2[illegible] minutes of infusion. The galactose content of the urine collected at this time was 95.0 mg., averaging 3.4 mg. per minute for the first 28 minutes. However, this average has little meaning because it is an average of urine which was formed both when galactose levels in blood were very low and galactose excretion in urine was negligible, and when levels in blood were higher and excretion in urine was appreciable. The galactose concentration of the subsequent 9 peripheral

blood samples, drawn over the next 45 minutes, varied only slightly from the 25 minute sample. The arithmetic mean of these values was 15.95 mg. per 100 ml., with a standard deviation of 0.79 mg. per 100 ml. (The arithmetic mean will be referred to henceforth as $P_{eq}$, the mean equilibrium peripheral concentration, and is shown as the dotted line in Fig. 1.) Urine collected during the equilibrium period, from 28 to 79 minutes, contained 290 mg. galactose, or a mean galactose of 5.7 mg. per minute. This average means more, because peripheral blood concentration was constant during the period of formation of this urine. During the equilibrium, the rate of disposal of galactose through all routes must have equalled the rate of infusion, since the peripheral blood level neither rose nor fell. In this instance, therefore, extraurinary, intracorporeal removal of galactose (R) was equal to 292 (I) – 6 (U) or 286 mg.

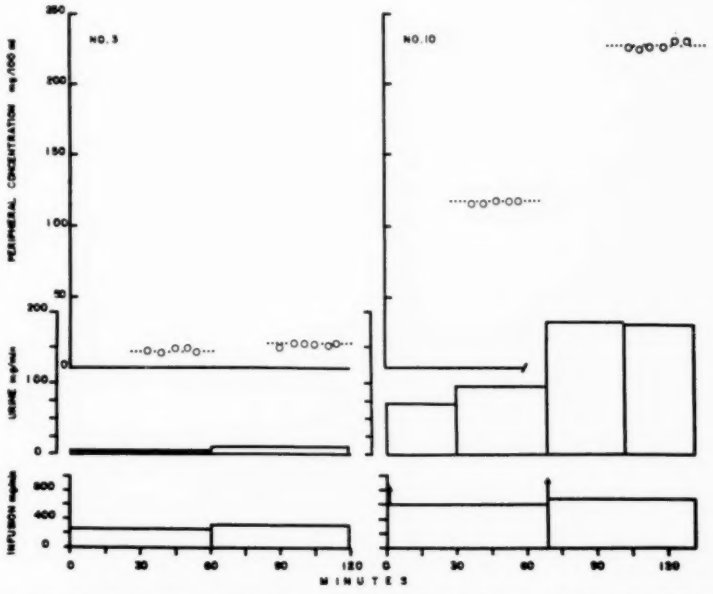


Fig. 2.—Typical results of galactose titration in 2 subjects. Left: in Subject 3, infusion of galactose at the rate of 244 and 302 mg. per minute respectively, resulted in little loss of galactose in the urine and low values for $P_{eq}$. Right: in Subject 10, infusion of galactose at the rate of 598 and 684 mg. per minute, respectively, resulted in large losses of galactose in the urine and high values for $P_{eq}$. The arrows indicate priming doses of 16 Gm. galactose each.

per minute. Renal clearance of galactose ($Cl_U$) $= \frac{5.7}{0.16} = 35.6$ ml. per minute. Finally, assuming for the moment that all intracorporeal removal was due to hepatic activity, "hepatic" clearance of galactose ($Cl_H$) $= \frac{286}{0.16} = 1{,}790$ ml. per minute.

Table I presents the data obtained in the next 12 subjects, in each of whom 2 levels of galactose infusion were done (galactose titration). Rates of infusion, mean equilibrium peripheral concentrations, standard deviations of P from $P_{eq}$, and urine excretions of galactose per minute, as well as the calculated

TABLE I. RESULTS OF GALACTOSE TITRATION IN 12 SUBJECTS WITHOUT LIVER DISEASE

| SUBJECT | PERIOD | PRIMING DOSE (GM.) | INFUSION RATE (MG./MIN.) | LENGTH OF INFUSION (MIN.) | AT EQUILIBRIUM | | | | CALCULATED | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | NO. OF SAMPLES | $P_{eq}$ ± S.D. (MG./100 ML.) | | U (MG./MIN.) | R (MG./MIN.) | $Cl_U$ (ML./MIN.) | $Cl_R$ (ML./MIN.) |
| | 1 | | 122 | 60 | 5 | 4.5 | 0.3 | | 122 | | 2710 |
| 1 | 2 | | 186 | 60 | 7 | 8.3 | 0.5 | | 186 | | 2250 |
| | Δ* | | *+64* | | | *+3.8* | | | *+64* | | *−560* |
| | 1 | | 213 | 50 | 5 | 7.2 | 0.4 | 6 | 207 | 85 | 2880 |
| 2 | 2 | | 321 | 51 | 4 | 12.3 | 1.1 | 13 | 308 | 108 | 2550 |
| | Δ | | *+108* | | | *+5.1* | | *+7* | *+101* | *+23* | *−380* |
| | 1 | 3.3 | 244 | 58 | 5 | 10.5 | 0.6 | 4 | 240 | 36 | 2280 |
| 3 | 2 | | 302 | 55 | 5 | 16.5 | 0.5 | 7 | 295 | 43 | 1790 |
| | Δ | | *+58* | | | *+6.0* | | *+3* | *+55* | *+7* | *−490* |
| | 1 | | 292 | 45 | 3 | 24.1 | 0.9 | 7 | 285 | 27 | 1180 |
| 4 | 2 | | 438 | 51 | 5 | 32.5 | 0.6 | 15 | 423 | 45 | 1300 |
| | Δ | | *+146* | | | *+8.4* | | *+8* | *+138* | *+18* | *+120* |
| | 1 | 3.6 | 292 | 68 | 6 | 16.8 | 1.0 | 12 | 280 | 74 | 1665 |
| 5 | 2 | | 438 | 55 | 6 | 33.8 | 2.2 | 22 | 416 | 67 | 1230 |
| | Δ | | *+146* | | | *+17.0* | | *+10* | *+136* | *−7* | *−435* |
| | 1 | 3.9 | 339 | 57 | 5 | 22.3 | 0.9 | 14 | 325 | 60 | 1460 |
| 6 | 2 | 4.9 | 508 | 50 | 5 | 53.2 | 1.0 | 41 | 467 | 76 | 880 |
| | Δ | | *+169* | | | *+30.9* | | *+27* | *+142* | *+16* | *−580* |
| | 1 | 5.8 | 416 | 61 | 6 | 30.6 | 0.0 | 14 | 402 | 46 | 1315 |
| 7 | 2 | | 482 | 60 | 5 | 42.5 | 1.4 | 34 | 448 | 80 | 1055 |
| | Δ | | *+66* | | | *+11.9* | | *+20* | *+46* | *+34* | *−260* |
| | 1 | 2.9 | 547 | 56 | 6 | 40.2 | 0.9 | 63 | 484 | 156 | 1200 |
| 8 | 2 | | 582 | 50 | 3 | 76.1 | 0.6 | 73 | 509 | 96 | 695 |
| | Δ | | *+35* | | | *+35.9* | | *+10* | *+25* | *−60* | *−505* |
| | 1 | 16.5 | 592 | 70 | 5 | 85.4 | 2.0 | 95 | 497 | 112 | 580 |
| 9 | 2 | 8.3 | 646 | 61 | 5 | 122.9 | 0.0 | 140 | 506 | 114 | 410 |
| | Δ | | *+54* | | | *+37.5* | | *+45* | *+9* | *+2* | *−170* |
| | 1 | 16.0 | 598 | 68 | 5 | 116.6 | 1.3 | 94 | 504 | 81 | 430 |
| 10 | 2 | 16.0 | 684 | 63 | 6 | 225.5 | 2.2 | 177 | 507 | 78 | 225 |
| | Δ | | *+86* | | | *+108.9* | | *+83* | *+3* | *−3* | *−205* |
| | 1 | 15.5 | 598 | 62 | 6 | 66.2 | 1.9 | 88 | 510 | 132 | 765 |
| 11 | 2 | 23.5 | 736 | 60 | 6 | 156.5 | 2.2 | 203 | 533 | 130 | 340 |
| | Δ | | *+138* | | | *+90.3* | | *+115* | *+23* | *−2* | *−425* |
| | 1 | 26.2 | 671 | 62 | | 131.3 | 1.8 | 159 | 512 | 121 | 390 |
| 12 | 2 | 8.7 | 710 | 50 | 5 | 172.5 | 1.0 | 201 | 509 | 117 | 295 |
| | Δ | | *+39* | | 4 | *+41.2* | | *+42* | *−3* | *−4* | *−95* |

*Δ = difference between periods (2 − 1).

values for R, $Cl_U$, and $Cl_H$ for both periods in each subject, are listed. The first rates of infusion varied from 122 to 671 mg. per minute. The second rates of infusion varied from 186 to 736 mg. per minute. The differences between paired infusions varied from 35 to 169 mg. per minute. The times for infusions varied from 45 to 70 minutes, with an average of 58 minutes per infusion. The number of observations, from which $P_{eq}$ was calculated, varied from 3 to 7, with an average of 5.1. The small standard deviations of P from $P_{eq}$ indicate that satisfactory equilibria were attained.

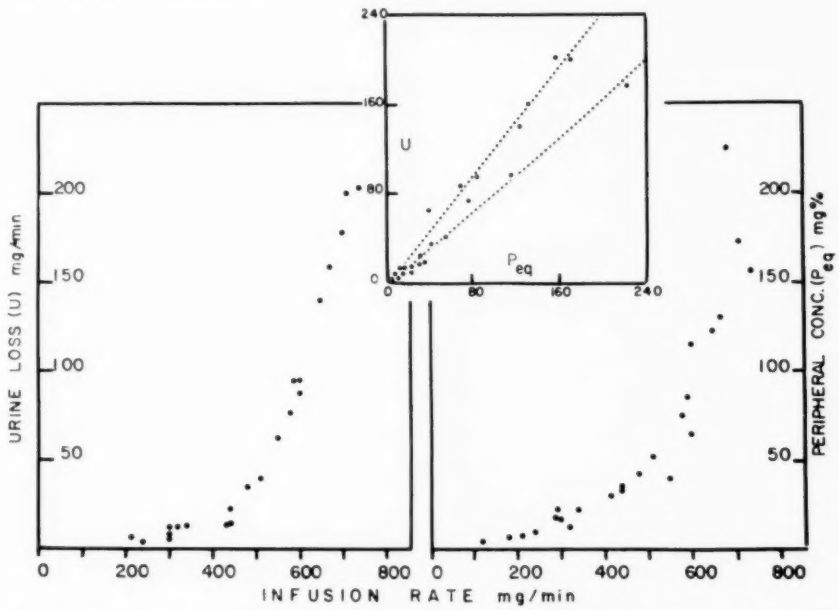


Fig. 3.—Relation of urine excretion rates and mean equilibrium peripheral concentrations to infusion rates of galactose. Inset: comparison of excretion rates to equilibrium concentrations. The upper dotted line represents a renal clearance of 120 ml. per minute, the lower dotted line, a clearance of 80 ml. per minute.

Representative of the results typical for the method are those plotted in Fig. 2. In Subject 3 (Fig. 2, left), the rate of infusion for the first period was 244 mg. per minute and for the second period 302 mg. per minute. U was 4 mg. per minute in the first period and 7 mg. per minute in the second. $P_{eq}$ was 10.5 and 16.5 for the respective periods. With both infusion rates well below maximum removal capacity, $P_{eq}$ and U were small. In Subject 10 (Fig. 2, right), by contrast, infusions at rates of 598 and 684 mg. per minute, respectively, effected much higher concentrations in the blood and excretions in the urine. The first infusion exceeded maximum hepatic removal capacity. The excess had to be eliminated in the urine in order to attain concentration equilibrium. At equilibrium, $P_{eq}$ was 116.6 mg. per 100 ml., and U was 94 mg. per minute. With the second infusion, the increment could not be removed intracorporeally and had to be eliminated in the urine to attain concentration equilibrium. Now, $P_{eq}$ was 225.5 mg. per 100 ml., and U was 177 mg. per

minute. Thus, in Subject 3, an increment of infusion of 58 mg. per minute effected a concentration increment of only 6.0 mg. per 100 ml., and a urine excretion increment of only 3 mg. per minute. In Subject 10, by contrast, an

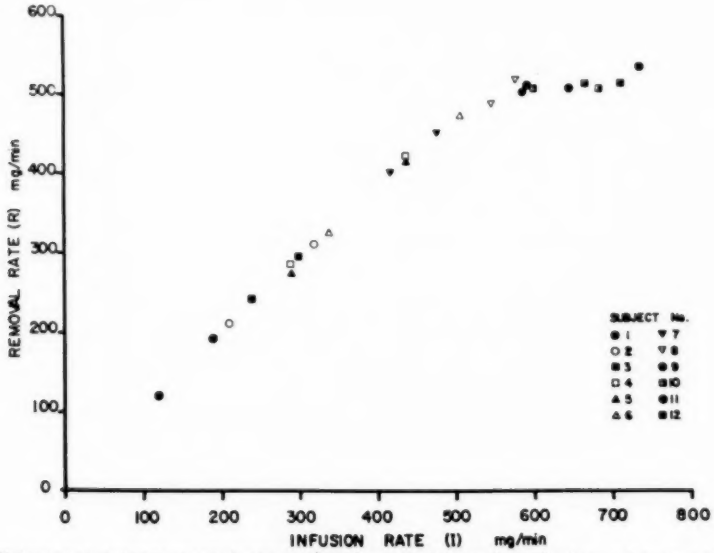


Fig. 4.—Calculated intracorporeal removal rate in relation to infusion rate of galactose in 12 subjects without liver disease.

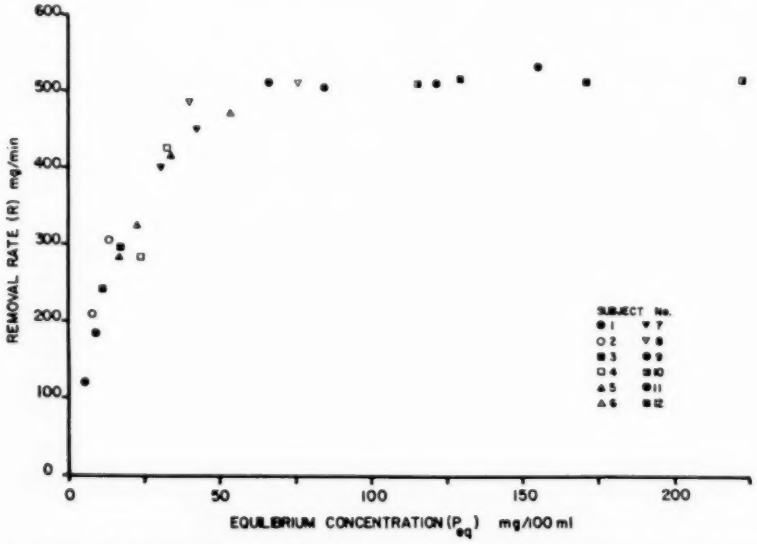


Fig. 5.—Galactose titration curve, derived from data obtained, in 12 subjects without liver disease. The coordinates differ from those classically used to plot titration curves (e.g., for the kidney) in that the abscissa is $P_{eq}$ instead of load per removal maximum ratio, and the ordinate is removal instead of removal per removal maximum ratio. Because removal maxima and loads could not be determined for each subject, these ratios could not be calculated for each individual. In the absence of extrahepatic removal, for an individual having a removal maximum of 500 mg. per minute and a hepatic blood flow of 1,000 ml. per minute, a removal per $L_m$ ratio of 1.0 on the ordinate would correspond to the point labeled 500, and a load per $L_m$ ratio of 1.0 on the abscissa would correspond to the point labeled 50.

infusion increment of 86 mg. per minute effected a concentration increment of 108.9 mg. per 100 ml., and a urine excretion increment of 83 mg. per minute. The latter thus accounted for more than 96 per cent of the increment infused, which indicates that further intracorporeal removal of galactose was not accomplished, despite the faster rate of infusion.

The graphs (Fig. 3) were obtained when U and $P_{eq}$ for each set of experiments were plotted against I. At rates of infusion up to 500 mg. per minute only small amounts of galactose were lost in the urine, and $P_{eq}$ increased only moderately with the increasing rates of infusion. At rates of infusion above 500 mg. per minute, excretion in the urine markedly increased, and equilibria were obtained at greatly increased peripheral concentrations. As illustrated in the inset of Fig. 3, U was, in general, proportionate to $P_{eq}$.

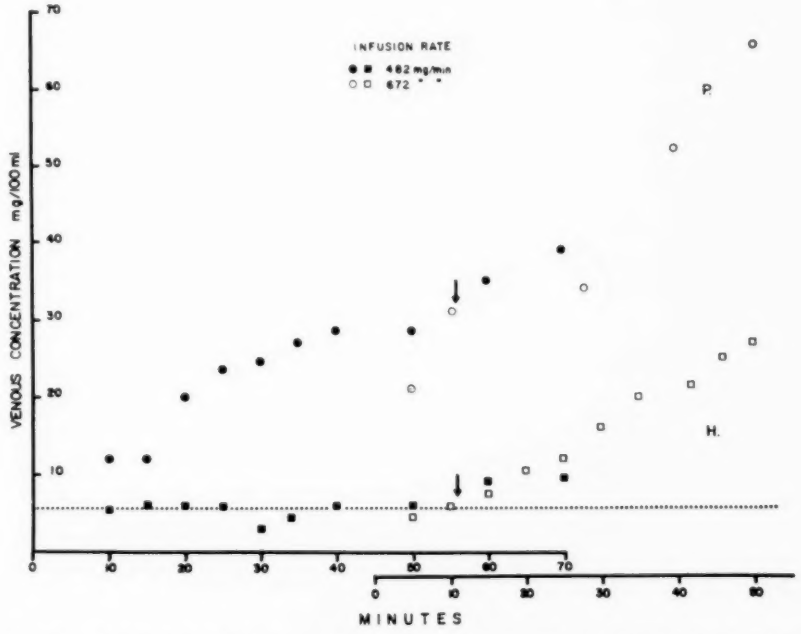


Fig. 6.—Peripheral (circles) and hepatic vein (squares) concentrations of galactose during constant infusion of galactose in 2 subjects of equal height and weight. No priming injections were given, and sampling was discontinued before concentration equilibrium was attained. The dotted line is an average of the initial hepatic vein concentrations. That these were not the zero expected by theory may have been due to sampling error caused by reflux from the vena cava or by arteriovenous shunts. The arrows indicate the time at which P first exceeded 30 mg. per 100 ml.

Fig. 4 shows a comparison between the calculated intracorporeal removal rate, R, and the rate of infusion. It can be seen that the removal rate was proportionate to the rate of infusion for all infusions up to approximately 580 mg. per minute. At faster rates of infusion, intracorporeal removal did not increase significantly above 500 mg. per minute. The attainment of a maximum removal rate was shown more strikingly in the plot of R against $P_{eq}$ (Fig. 5). Removal rate increased proportionately to the equilibrium concentration, until

the latter was approximately 70 mg. per 100 ml., and R was about 500 mg. per minute. At all concentrations above this level (the highest being 225.5 mg. per 100 ml.), intracorporeal removal did not significantly exceed 500 mg. per minute. It should be noted that the ascending portion of the curve, shown in Fig. 5, joins the horizontal portion by a gradual smooth curve, rather than by an acute angle.

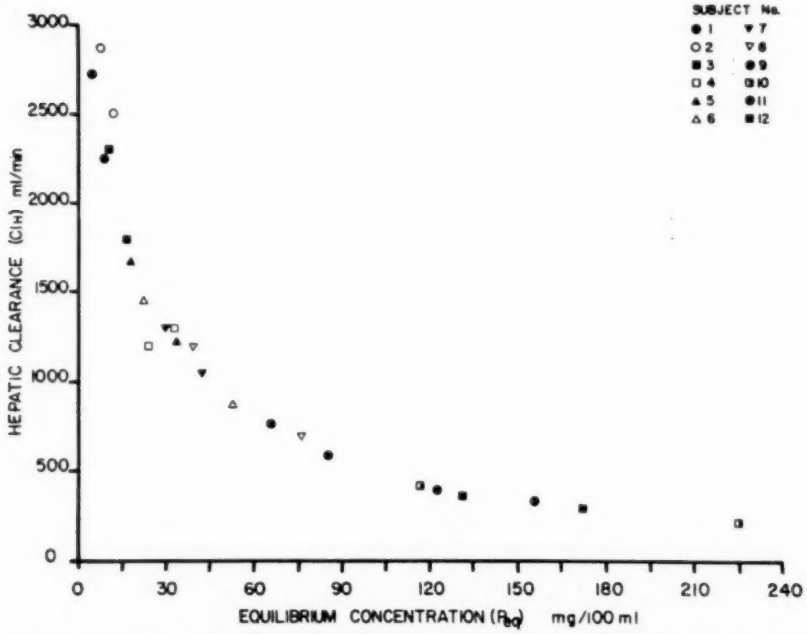


Fig. 7.—Relation of calculated "hepatic" clearance of galactose to $P_{eq}$ in 12 subjects without liver disease. In all but 1 subject (No. 4) an inverse relation between concentration and clearance was found. The fact that this relationship holds as well for the range of $P_{eq}$ below 30 mg. per 100 ml. suggests the presence of a small amount of extrahepatic removal of galactose.

Hepatic vein catheterization was performed in conjunction with galactose infusion in 2 subjects, who were of similar height and weight. In each, priming doses were not given, and blood from both peripheral (P) and hepatic (H) veins was sampled frequently, in order that the appearance of galactose in the hepatic vein in relation to its concentration in the periphery (Fig. 6) could be studied. In each subject, the first hepatic vein concentrations obtained were not truly zero, but were 5.4 mg. per 100 ml. This base line value is shown as the dotted line in Fig. 6. In the first subject (solid symbols), infused at a rate of 484 mg. per minute, H did not exceed the initial value in the 8 samples drawn during the first 55 minutes of infusion. During this period, P was less than 30 mg. per 100 ml. When P exceeded this concentration (arrow), H first rose above the base line. In the second subject (open symbols), a faster galactose infusion, at a rate of 684 mg. per minute, was given. P rose much faster and exceeded 30 mg. per 100 ml. in 10 minutes. It was at this time that galactose concentrations, greater than the baseline, first appeared in blood from the

hepatic vein. H increased steadily thereafter in keeping with the rising P. However, the slope of the ascending curve of H was shallower than that of P, and even at the termination of the experiment the 2 curves were not parallel.

The curve (Fig. 7) was obtained when intracorporeal clearance ("hepatic" clearance) of galactose was calculated for each pair of infusions in each subject, and the results plotted against $P_{eq}$. In all, with the exception of Subject 4, an inverse relation between clearance and equilibrium concentration was found at all levels of $P_{eq}$, including those below 30 mg. per 100 ml.

## DISCUSSION

It is generally agreed that substances move across biologic membranes by simple diffusion, facilitated diffusion, or active transport.[1] Active transport implies that a substance is transferred across these membranes against a concentration gradient by using energy derived from cellular metabolism. However the gradient may be difficult to demonstrate, and the other mechanisms of transfer may appear to have an effect on this. Other important features of active transport are that the transfer is unidirected and that there is a nonlinear relation between rate of transfer and concentration, so that eventually saturation of the transport system occurs, and a fixed maximal rate of transfer is attained. The renal tubular epithelium has been shown to utilize active transport, for transfer of a number of substances. Determination of the maximal rate of transfer ($T_m$) for tubular reabsorption of some (e.g., glucose), and tubular secretion of others (e.g., paraaminohippurate), has become a standard procedure in the study of renal physiology and pathology.[2]

The role of active transport, and particularly of transfer maxima, by the liver epithelial cells has not been so clearly defined or demonstrated. Mason and co-workers,[3] Lewis,[4] and Verschure[5] have each shown that the liver has a limited capacity for the uptake of sulfobromophthalein (BSP) from the blood, and have calculated the maximum rate from known rates of infusion and changing peripheral blood concentrations. By analogy to the term $T_m$ for tubular mass, the term $L_m$ was proposed for "functional hepatic mass."[3] Although $T_m$ may also be taken to stand for "transport maximum" and thereby be perfectly suitable to the liver, as well as to the kidney or to any other tissue, its usual identification with renal tubular maxima makes the use of distinct designations for other organs desirable. Since $L_m$ has been previously used for the liver, its use has been continued in the present study.

When a substance is infused intravenously at a constant rate, concentration equilibrium is attained only when the rate of disposal by all means is equal to the infusion rate, for if disposal is slower than infusion, the concentration will rise, and if it is faster, the concentration will fall. Infusion at a constant rate to concentration equilibrium not only affords a means of easily measuring disposal rate, but avoids, as nonequilibrium states do not, estimation of such factors as volume of distribution, rate of diffusion, and rapidity of mixing, which may be very difficult, if not impossible, to measure. Berger and co-workers[11] have indicated that even constant infusion studies may be misleading, and the

apparent equilibrium may be an illusion. Within the bounds of practical clinical studies, however, this technique seems to avoid many pitfalls, and is the best available. It was used, therefore, in the present study. Galactose was chosen to be the substance used to study $L_m$ in the liver for several reasons. Repeated clinical experience has shown that galactose removal from the blood depends primarily upon hepatocellular function.[12, 13] Recently, the liver cell has been shown to contain a specific enzyme system which converts galactose to glucose.[14] Enzyme systems are often a feature of active transport, and renal transport, at least, often conforms to the behavior predicted on the basis of enzyme-substrate interactions.[1] Although the red blood cell and the brain also contain some parts of the same enzyme system,[15] the liver has been shown to be the major site, quantitatively, of galactose uptake.[16] Finally, the observed behavior of galactose after single intravenous injection suggested the presence of a removal process with a maximum limiting rate.[6]

To demonstrate the $L_m$, galactose titration was employed. By titration is meant the measurement of removal at successively increased loads to determine the load at which maximum removal is reached. Smith and co-workers[7] used the titration technique to demonstrate reabsorptive maxima for glucose by the renal tubule. They were able to titrate each subject with many different loads. In the present study each galactose load was infused for an extended period (average 58 minutes) to assure adequate equilibrium. It was not practical, therefore, to do more than 2 infusions for each subject, as this would have extended the procedure to intolerable lengths. Nevertheless, the data from the 24 infusions into 12 subjects (Figs. 4 and 5) were in close agreement, and the individual values deviated very little from a single curve.

When the removal rate (R) was compared to rate of infusion (Fig. 4) and to equilibrium concentration ($P_{eq}$) (Fig. 5), it was found that intracorporeal removal did not significantly exceed 500 mg. per minute. Note that in Fig. 5 the abscissa is $P_{eq}$, not load. Because blood flow was not simultaneously measured, load (which is the product of flow times concentration) could not be known exactly. It is load rather than concentration with which we are concerned, for it is necessary to know that load was excessive and that every opportunity for maximal removal was afforded, in order to conclude that a maximum removal capacity was shown. A few simple calculations assure that load was indeed well in excess of removal capacity in these experiments. For blood galactose concentrations between 70 and 225 mg. per 100 ml., the maximum intracorporeal transfer rate was 500 mg. per minute. Hepatic removal by itself could, therefore, have been no greater. If hepatic blood flow was 1,500 ml. per minute (the average normal), then load varied from 1,050 to 3,375 mg. per minute, and load: removal maximum ratios varied from 2.10 to 6.75 in this range of concentration. If flow was as low as 500 ml. per minute (quite extreme for normals), load: removal maximum ratios varied from 0.70 to 2.25. If flow was as high as 3,000 ml. per minute, ratios varied from 4.20 to 13.50. It seems reasonable to assume, therefore, that the load presented to the liver was well in excess of

hepatic removal capacity at all concentrations corresponding to the horizontal portion of the curve (Fig. 5). Similar reasoning can be applied to all other possible, though quantitatively less important, sites of galactose removal.

The fact that intracorporeal removal did not increase, despite increasing loads, demonstrates the presence of a fixed maximum transfer (removal) rate, presumably caused by saturation of the transport system involved. The presence of a total body maximum removal rate implies that each of the sites effecting removal individually has a maximum rate, for if the removal rate of even one site was unlimited, total body removal would be unlimited. A body of evidence attests to the fact that the liver is the major site of removal of galactose from the blood. The liver thus appears to have a maximum capacity for removal of galactose.

Extrahepatic, intracorporeal removal is also likely. In animals, which have undergone hepatectomy, nephrectomy, and evisceration, galactose disappears from the blood, although at a slow rate.[17] Studies in which radioactive $C^{14}$ galactose has been used have demonstrated metabolism of this compound by tissues other than the liver.[18] The results of the present study are also in keeping with a small amount of extrahepatic removal of galactose. By hepatic vein catheterization, it was shown that significant galactose did not appear in the hepatic vein until P reached 30 mg. per 100 ml. (Fig. 6). When P was greater than 70 mg. per 100 ml. there was constant removal of galactose (Fig. 5). There were, therefore, progressively smaller extraction ratios for rising values for P. Given a constant hepatic blood flow, clearance can be expected to have decreased with increasing values for P above 30 mg. per 100 ml. Below this concentration a constant hepatic clearance equal to hepatic blood flow could be expected, if the liver were the sole site for galactose removal. Examination of the plot of clearance against $P_{eq}$ (Fig. 7) shows an inverse relation of clearance to $P_{eq}$ at all levels, including those under 30 mg. per 100 ml. One explanation for this finding is the presence of a small, but constant, amount of extrahepatic removal.*

*Assume that intracorporeal removal, R, is caused by both liver removal, $L_r$, and extrahepatic removal, $E_r$. Further assume that $E_r$ has a maximum capacity ($E_m$) that is 10 per cent of total intracorporeal removal capacity, and that liver maximum capacity ($L_m$) is 90 per cent of intracorporeal capacity. If R maximum is 500 mg. per minute, then $E_m$ is 50 mg. per minute, and $L_m$ is 450 mg. per minute. If hepatic blood flow (HBF) is 1,500 ml. per minute, load (flow × concentration) will exceed $L_m$ at P > 30 mg. per 100 ml. As P is decreased below this concentration, load to the liver will become progressively smaller and less than $L_m$, and complete removal will be effected. Because of the magnitudes, however, of extrahepatic flow and $E_m$, load to these sites will exceed $E_m$ at almost any measurable P. Hence, as P is decreased, $L_r$ will be proportionately decreased, whereas $E_r$ will be constant and at its maximum, $E_m$. Since $HBF = \frac{L_r}{P-H}$ (Fick), and $Cl_H = \frac{L_r}{P}$, when H is zero, $Cl_H$ = HBF. Thus, when P is between 1 and 30 mg. per 100 ml. and H is zero, $Cl_H$ should be constant and equal to HBF. But if R is really $L_r$ plus $E_r$, then, substituting R for $L_r$ in the clearance equation will result in a progressively larger overestimate of $L_r$ as P is lowered, because of the progressively larger contribution to R made by $E_m$. In the example cited, when P = 30 mg. per 100 ml. using $L_r$, $Cl_H = \frac{450}{0.30} = 1{,}500$ ml. per minute, but apparent clearance, using R instead of $L_r$, is: $Cl_H = \frac{500}{0.30} = 1{,}666$ ml. per minute, an estimate that is 111 per cent of the true value. When P = 5 mg. per 100 ml., $L_r$ = 75, and $E_m$ = 50 mg. per minute. Using $L_r$, $Cl_H = \frac{75}{0.05} = 1{,}500$ ml. per minute, but using R, $Cl_H = \frac{125}{0.05} = 2{,}500$ ml. per minute, an estimate that is 167 per cent of the true value.

The theoretical plot of concentration (or load) against removal of a substance transferred by active transport shows increasing removal with increasing load, until the maximum transfer rate is reached. At this point of saturation of the transport system, the ascending straight line has an abrupt point of inflection, and meets the horizontal line of constant removal. Departure from this ideal curve, with a gradual transition from the ascending to the horizontal portion, instead of an abrupt, acute angle, has been termed splay.[7] The significance of splay in titration curves of renal tubular function has been discussed extensively.[7, 19, 20] The significance of splay in the galactose titration curve (Fig. 5) is, for the present, unknown. In part, it may be due to the fact that the curve was derived from galactose titration of 12 subjects. Another possibility is that there exists a range in the liver, as in the kidney, wherein saturation of the transport mechanism is completed in some areas before it is completed in others. This would give rise to a minimum threshold, below which no area spills galactose, as well as a maximum threshold, above which all areas spill galactose. From the data of the present study, the minimum threshold can be expected to be near 30 mg. per 100 ml. and the maximum near 70 mg. per 100 ml. As shown by catheterization, galactose does not appear in the hepatic vein in significant amounts below a peripheral concentration of 30 mg. per 100 ml. (Fig. 6).[21, 22] This can be taken to mean that no area of the liver is saturated and, therefore, no spill occurs. Above 70 mg. per 100 ml., maximum removal is reached (Fig. 5). Hence, all areas must be saturated and be spilling. It can be supposed that between 30 and 70 mg. per 100 ml. some areas are saturated and some are not. This is partly supported by the fact that up to peripheral levels of 68 mg. per 100 ml., the slope of hepatic vein galactose appearance is less steep than the slope of rising peripheral concentrations (Fig. 6). However, further studies will be necessary before the interpretation of these findings can be made with any confidence.

## SUMMARY

Evidence for the existence of a maximum removal capacity ($L_m$) for galactose in the human liver has been obtained from the results of galactose titration in each of 12 subjects, who had no liver disease. Intracorporeal removal of galactose was calculated from the known rate of infusion, and from the measured rate of excretion in urine during the period of blood concentration equilibrium, for each of 2 infusions in each subject. At equilibrium, excretion of galactose in urine was proportionate to its peripheral blood concentration.

From the data, a titration curve can be constructed which primarily represents hepatic transport of galactose. It is splayed from the theoretical concentration–transfer rate curve. The maximum intracorporeal removal capacity for galactose is normally approximately 500 mg. per minute. Although the greatest part of this removal capacity is hepatic, a small amount of extrahepatic removal probably occurs.

Saturation methods appear to be applicable to the study of the liver and to afford another means of assessing hepatocellular function.

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# MEASUREMENT OF BODY RADIOACTIVITY FOR STUDIES OF SODIUM METABOLISM

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THE metabolic behavior of radioactive tracer elements and tagged compounds in man has been studied in the past mainly by in vitro measurements of the radioactivity of biologic samples, such as serum, red cells, excreta, or excised tissues. In vivo measurements have been limited mainly to the external counting of accumulation and discharge of gamma ray emitting radioactive elements and tagged compounds which tend to concentrate in specific organs or body sites, such as thyroid, kidneys, liver, spleen, or bone marrow. Recently, it has become possible to extend in vivo techniques to include the measurement of gamma radiations emitted from radioactive tracers which are distributed throughout body tissues, without marked concentration in any particular area. This has been made possible by the development of external counting systems that are capable of recording the gamma rays emitted from the whole, or at least the major part, of the human body.[1]

An important application of body counting is the long-term investigation of sodium metabolism with the long half-life (2.6 years) gamma ray emitter $Na^{22}$ used as a tracer. In the past, changes in body content of $Na^{22}$ after the administration of a known tracer dose have been determined either by measuring urinary $Na^{22}$ excretion or by following changes in serum $Na^{22}$ concentration. It has been shown, however, that agreement between these 2 methods is often poor, and that even in normal subjects, the observed (apparent) half-time for urinary excretion of $Na^{22}$ may be 2 or 3 times the half-time for fall off of serum concentration.[2] With body radioactivity counters, it is now possible to measure changes in body content of $Na^{22}$ directly.[3, 4]

A body radioactivity counter which has been designed for clinical tracer studies will be described, its gamma ray counting characteristics will be discussed, and an example of a $Na^{22}$ study in a normal subject will be presented.

## MATERIALS AND METHODS

*Body Radioactivity Measurements.*—A single large sodium iodide crystal (4 inches in diameter and 4 inches high) in a copper container with a quartz window has been used for

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radioactivity measurements of the body. The sodium iodide crystal, with its photomultiplier tube, is enclosed in a cylindric 5-inch-thick steel shield which is open at one end (Fig. 1). All pulses from the crystal are counted as a function of pulse height with a 256-channel pulse height analyzer.*

The subject is seated in a contour chair beneath the open end of the shield, and the center of the crystal is positioned at 50 cm. from the midline of the body (Fig. 2). In this position the crystal measures mainly the radioactivity from the subject's trunk, as is shown by the relative counting rates of a point source in air at the positions indicated in Fig. 2. Increasing counting losses with increasing angle from the center axis of the crystal are due to absorption in the shield which surrounds the crystal. The absorption of the gamma rays by the patient's body has not been taken into account, in Fig. 2.

With the sodium iodide crystal inside the 5-inch-thick steel shield, the main contribution to the background counting rate is the natural radium and potassium radioactivity in the concrete floor and the asphalt tiles beneath the counter. When the area under and around the contour chair is covered with low radioactivity Dunite bricks† and lead (Fig. 2), the background counting rate is markedly reduced.

*Sample Measurements.*—The radioactivities of serum and urine samples were determined in a NaI(Tl) well-counter. Chemical sodium concentrations of these samples were measured by flame photometry.‡

*Clinical Study.*—A healthy man received intravenously a 12 $\mu$c $Na^{22}$ tracer dose. For the first 7 days after the $Na^{22}$ injection, he took his normal diet, which had a variable sodium content. During the next 11 days he took a liquid synthetic diet which provided not more than 1 mEq. of sodium per day.§ During the following 8 days, the subject continued the liquid diet, with the addition of an intravenous infusion of 235 mEq. of sodium chloride in the form of 5 per cent saline solution. During the final 12 days, the subject again took his normal diet, which had a variable sodium content.

Urine collections were made from one morning to the next, and were ended when the patient was in a relatively fasting state. Every 1 to 4 days, venous blood samples were taken after the urine collection was made.

Radioactivity measurements of the body were made twice daily (morning and afternoon), after the subject rested in the contour chair for 15 minutes. Only the morning measurements were used in the calculations described in the next section; the afternoon measurements served as a check.

## CALCULATIONS

The fraction of the initial $Na^{22}$ tracer dose, which remained in the subject after each urine collection period was determined both by counts of radioactivity of the body and by measurements of urinary excretion of $Na^{22}$. Since uniform distribution of radiosodium throughout the body requires at least 15 hours in the normal subject,[5] and since some excretion of the radiosodium occurs during this period, a count of radioactivity of the body, which truly represents 100 per cent of the $Na^{22}$ dose, is not obtainable. It was assumed, therefore, that the urinary excretion of $Na^{22}$ for the first 24 hours accounted for the total $Na^{22}$ loss during that period, and that the count of radioactivity of the body at the end of the first 24 hours corresponded to the retention in the body which was determined from the urine measurements.

Exchangeable sodium ($Na_e$) was calculated from the formula:

$$Na_e = \frac{\text{Body } Na^{22} \text{ retention (per cent)}}{\text{Serum } Na^{22} \text{ concentration (per cent per liter)}} \times \text{Serum sodium concentration (mEq. per liter)},$$

where $Na^{22}$ retention in the body and $Na^{22}$ concentration in the serum were expressed as percentages of the initial dose. Exchangeable sodium was calculated both from retention determined by the body counter and from urine data.

*Radiation Counter Laboratories, Inc., Skokie, Ill.
†Harbison-Walker Refractories Co., Pittsburgh, Pa.
‡Flame Photometer Model 146, Perkin-Elmer Corp., Norwalk, Conn.
§The major components of the diet were supplied as Product 3060-1-A by Mead Johnson & Company, Evansville, Ind. The diet contained only 1,500 calories per day, because the subject wished to reduce his weight.

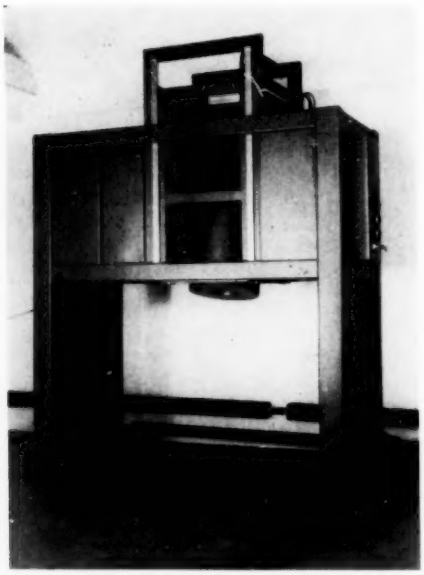

Fig. 1.—Mounting of 5-inch-thick steel shield for 4 by 4 inch NaI(Tl) crystal. The 1,200 pound shield has an outside diameter of 16 inches and a height of 20 inches. By motor it can be raised and lowered, or moved from right to left. The steel shield rotates around a horizontal axis, so that it can be directed at any angle. The whole mounting unit runs back and forth on tracks (not shown in figure).

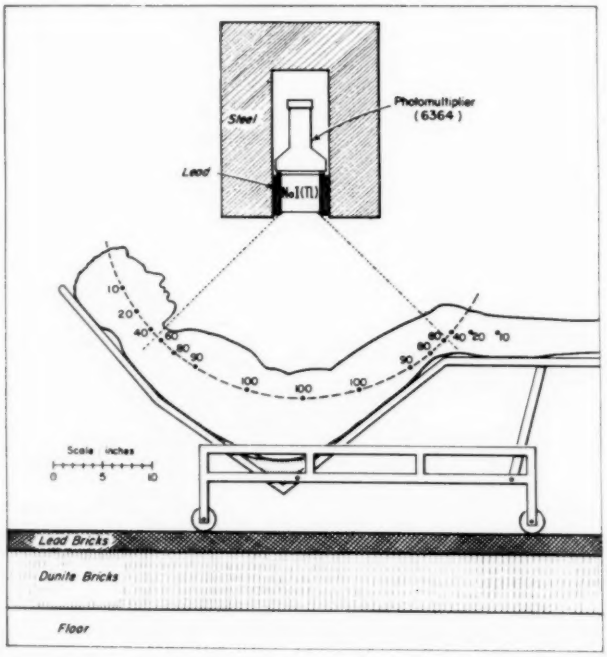


Fig. 2.—Position of subject in contour chair beneath 4 by 4 inch NaI(Tl) crystal. Contour chair rests on 6 inches of Dunite and 2 inches of lead bricks. The midline of the subject is about 50 cm. from the center of the crystal. The numbers refer to the relative counting rates in the 1.28 mev. photopeak from a $Na^{22}$ point source in air at the positions indicated.

## RESULTS

*Body Radioactivity Counter.—*

*Background counting rate:* Since the gamma rays emitted by most radioisotopes have energies between 0.2 and 2.0 mev., the background counting rate for this energy interval was determined. For the NaI(Tl) crystal setup described above, about 1,000 c.p.m. were recorded with energies between 0.2 to 2.0 mev. This is about 3 times the background counting rate observed by others with a 4 by 4 inch sodium iodide crystal completely shielded inside a steel room.[6, 7]

*Gamma ray counting efficiency:* The counting efficiency of a sodium iodide crystal for the gamma rays emitted from a small source at a given distance from the crystal center depends on the diameter and thickness of the crystal, as well as on the energy of the gamma rays. The total number of counts of all energies obtained from a point source on the axis of the crystal at 50 cm. from the center of a 4 by 4 inch sodium iodide crystal is shown in Fig. 3. The total counts per minute are plotted as a function of the gamma ray energy for a 1 $\mu$c source, with the assumption that 1 gamma ray is emitted per disintegration.

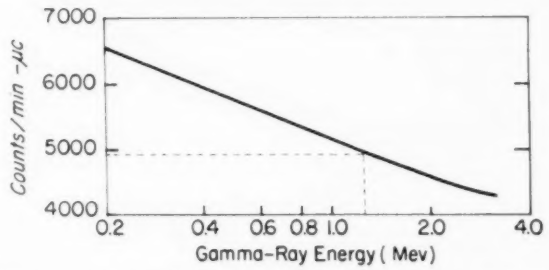


Fig. 3.—Gamma ray counting efficiency of 4 by 4 inch NaI(Tl) crystal as a function of gamma ray energy for a small source on the center axis, at 50 cm. from the center of the crystal. The counts per microcurie in 1 minute are given with the assumption that 1 gamma ray of a given energy is emitted per disintegration. The data were obtained by extrapolating to larger distances the values calculated for a crystal 5 inches in diameter and 4 inches thick[5] corrected to the crystal 4 inches in diameter.

The counting rate from a 1 $\mu$c $Na^{22}$ source at 50 cm. from the center of the crystal can be determined from Fig. 3. In 90 per cent of its disintegrations, $Na^{22}$ emits a 1.28 mev. gamma ray which results in a counting rate of $0.9 \times 4{,}900 = 4{,}410$ c. p. m.*

*Photopeak efficiency:* Gamma rays can be absorbed in the sodium iodide crystal by Compton interaction, pair production, or photoelectric effect. In the case of either Compton interaction or pair production the resulting pulse will have less energy than that corresponding to the energy of the incident gamma ray. A pulse corresponding to the energy of the primary gamma ray is obtained if the gamma ray is absorbed either by photoelectric effect or by Compton interaction followed by absorption of the scattered secondary gamma ray.

The probability of photoelectric absorption in the sodium iodide crystal decreases with increasing gamma ray energy independent of the size of the crystal. With crystals of increasing dimensions, the chance of total gamma ray

*In the present considerations, the additional counts at energies below 0.511 mev. (see spectra, Fig. 5) caused by the positron annihilation radiation can be disregarded.

absorption, by Compton effect and subsequent absorption of the scattered radiation, increases for all gamma ray energies. For the 4 by 4 inch NaI(Tl) crystal the photopeak efficiency is close to 100 per cent for all gamma rays with energies below 250 Kev. (Fig. 4). For the high energy (1.28 mev.) gamma rays of $Na^{22}$, only 44 per cent of the pulses appear in the photopeak area.

The background count for the photopeak energy interval of the 1.28 mev. gamma rays is about 40 c. p. m. By dealing only with the counts within the photopeak area, the counting rate caused by the 1.28 mev. gamma rays is reduced by a factor of 2.3, and the background counting rate is reduced by a factor of 25.

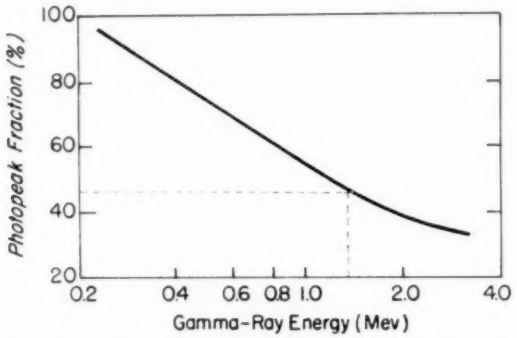


Fig. 4.—Fraction (per cent) of the total number of pulses that fall within the photopeak area, as a function of the energy of the incident gamma rays. Data are given for a broad parallel beam of gamma rays incident on the 4 by 4 inch NaI(Tl) crystal.[9] It is assumed that the same data hold for the gamma rays emitted from a small source at 50 cm. from the center of the crystal.

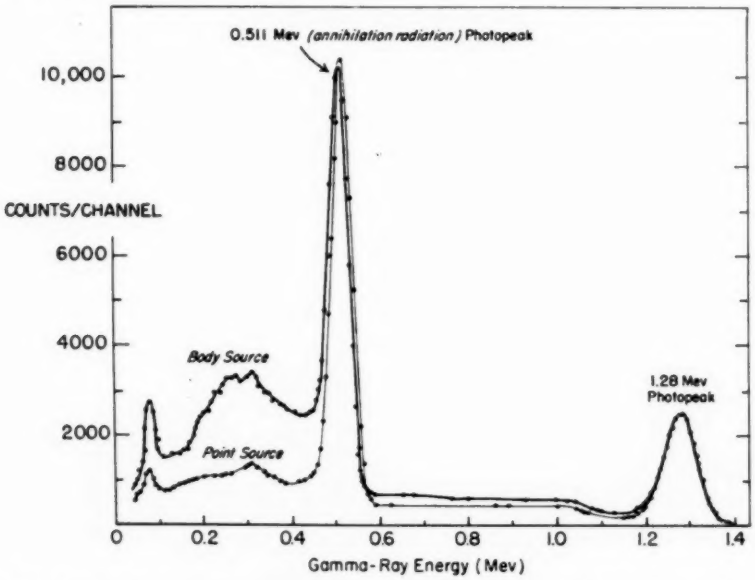


Fig. 5.—Gamma ray spectra from a small $Na^{22}$ source and from a subject containing $Na^{22}$. The 2 spectra are normalized at the 1.28 mev. gamma ray peak. The data were obtained with a 1/16 inch lead foil in front of the face of the NaI(Tl) crystal. Besides the photopeak of the 1.28 mev. gamma rays, another peak at 0.511 mev. is observed which results from $Na^{22}$ positron annihilation radiation. Comparing the 2 spectra, an increase in scattered radiation from the large source is observed.

*Counting of extended sources:* The counting efficiency for an extended source such as the human body is less than that for a small source. The total counting rate for a 1 $\mu$c $Na^{22}$ source was determined above as 4,410 c. p. m. and the photopeak efficiency as 44 per cent for the 1.28 mev. $Na^{22}$ gamma rays. Therefore, $4{,}410 \times 0.44 = 1{,}940$ c. p. m. which should fall within the photopeak area per microcurie of $Na^{22}$. Since 1 $\mu$c of $Na^{22}$ emits $2.0 \times 10^6$ gamma rays per minute, the actual photopeak efficiency at 50 cm. should be $1{,}940/2.0 \times 10^6 = 0.001$ counts per gamma ray, or 0.1 per cent of the emitted gamma rays. By direct measurements with a small liquid $Na^{22}$ source, a value of 0.11 per cent was observed.

When an average-sized man lies in the position illustrated in Fig. 2, the observed photopeak counting efficiency of $Na^{22}$ after equilibration in the body is 0.04 per cent, rather than 0.11 per cent, for the following reasons. First, as shown in Fig. 2, the head and the lower extremities are practically excluded from the measurements. Second, there are absorption and scattering of the gamma rays in the body. In passing through 12 cm. of tissue about ½ of the gamma rays undergo an interaction and are, therefore, lost from the photopeak area (Fig. 5). These effects reduce the photopeak efficiency by about a factor of 3 as compared with counting a small $Na^{22}$ source.

Eight hundred counts per minute are obtained in the photopeak area from a subject retaining 1 $\mu$c of $Na^{22}$. Since the background counting rate within the 1.28 mev. gamma ray photopeak area is 40 c. p. m., 0.1 $\mu$c $Na^{22}$ still yields a net counting rate of twice background. This latter dose represents the lower limit that can be counted with the present setup.

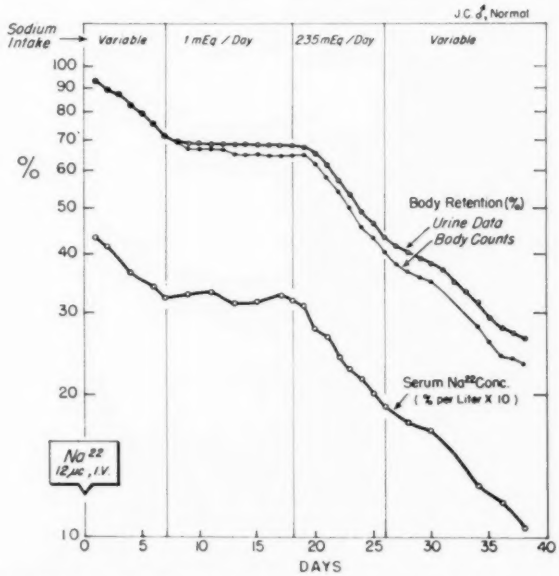


Fig. 6.—Retention of $Na^{22}$ in the body determined from urine data and counts of radioactivity in the body is plotted against time. There are increasing differences between the 2 curves, with less retention shown by the counts of radioactivity in the body. $Na^{22}$ concentration in serum is also plotted. The slopes of all the curves vary with sodium intake.

*Body Sodium$^{22}$ Retention.*—As the study proceeded, an increasing difference was noted between $Na^{22}$ retention in the body, based on urine data and on counts of radioactivity in the body with the latter showing the lesser retention (Fig. 6). This difference reached 12.7 per cent of the lower value at the end of

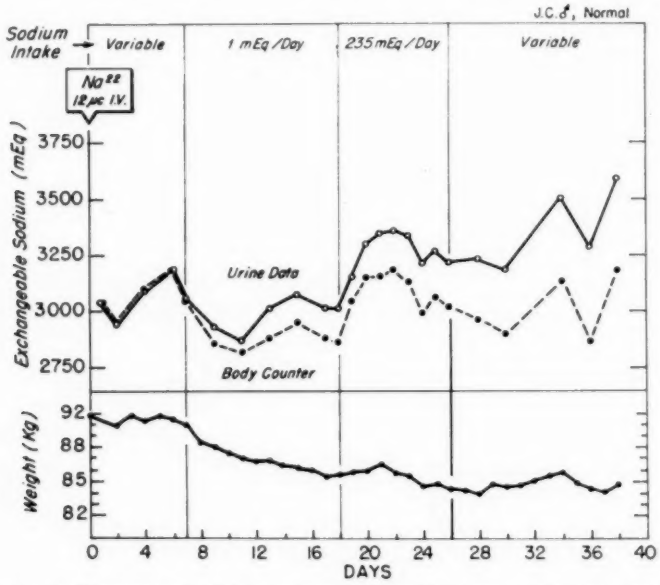


Fig. 7.—Exchangeable sodium values calculated from urine data and counts of radioactivity in the body are plotted against time. The subject's weights are also shown. Differences between exchangeable sodium values calculated by the 2 methods increase with time. Exchangeable sodium levels vary with sodium intake.

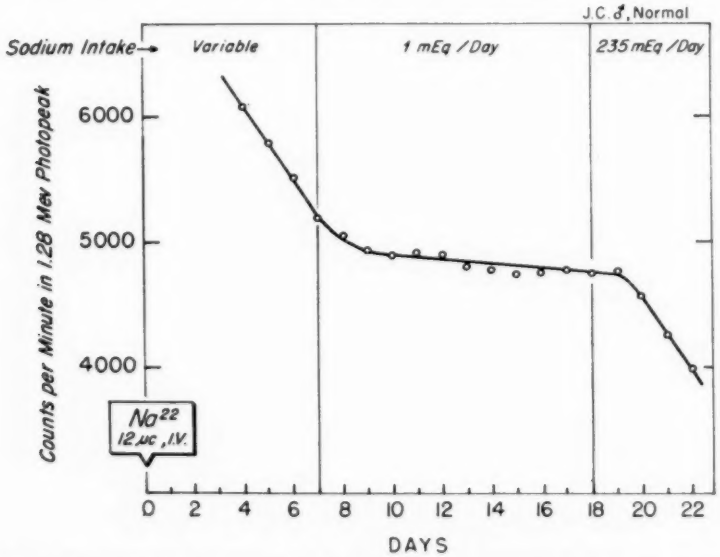


Fig. 8.—Counts per minute in the 1.28 mev. photopeak area from the subject during the period of low sodium intake. The maximum deviation from the assumed average curve is less than 2 per cent.

the 38 days (26.6 per cent retention by urine data; 23.6 per cent retention by counts of radioactivity in the body), and was reflected by a 410 mEq. difference in the values for exchangeable sodium calculated by the 2 methods (3,590 mEq. by urine data; 3,180 mEq. by counts of radioactivity in the body) (Fig. 7). In other similar studies, values for exchangeable sodium, calculated from counts of radioactivity in the body, were also smaller than values calculated from urine data. The latter became unreasonably high as the studies proceeded.

When the 1 mEq. sodium diet was substituted for the subject's normal diet, the exchangeable sodium calculated from counting data of radioactivity in the body decreased from 3,040 mEq. on the last day of the normal diet to an average value of 2,880 mEq. (5.3 per cent decrease) (Fig. 7). The subsequent change to a high sodium intake resulted in an increase from 2,880 mEq. to an average value of 3,090 mEq. (7.3 per cent increase). There were actually 2 phases evident in the change from the low to the high sodium intake. The exchangeable sodium increased to a maximum of 3,180 mEq. at the end of the fourth day of high sodium intake and then decreased to an intermediate level.

The observed half-times for urinary excretion of $Na^{22}$ and for fall off of body $Na^{22}$ activity and serum $Na^{22}$ concentration were dependent upon the level of sodium intake. The greater the sodium intake, the shorter the half-times. Within each dietary period the half-times for serum concentration and body $Na^{22}$ activity were the same, while the half-time for urinary excretion was slightly longer.

## DISCUSSION

For clinical tracer studies, the flexible arrangement of the shielded gamma ray detector, which can accommodate all kinds of patients with a minimum of discomfort and inconvienience, has proved satisfactory. With this body radioactivity counter it was possible to perform long-term studies of sodium metabolism accurately. It proved to be more effective than the usual urine collection method, because it accounted for extrarenal as well as renal losses of $Na^{22}$. These extrarenal losses are usually small during any given day. Stool losses of $Na^{22}$ in another normal subject studied in this laboratory averaged 0.017 per cent per day of the remaining $Na^{22}$ while the subject was on a very low sodium intake and 0.021 per cent per day during a high sodium intake period. Others have reported stool losses of $Na^{22}$ which average 0.06 per cent per day of the $Na^{22}$ dose on normal and salt-restricted diets, and sweat losses of $Na^{22}$ of 0.1 per cent per day on a salt-restricted diet.[10, 11] Since these small losses are cumulative, they become significant during prolonged studies; moreover, these extrarenal losses may be increased by intervening episodes of excess exercise, fever, or diarrhea. Even if there were no extrarenal losses, the body radioactivity counting method would still be preferable, because it obviates the necessity for an attempt to obtain complete urine collections.

In this and in other studies the half-times for urinary excretion of $Na^{22}$ were slightly prolonged as compared with the half-times for fall off of $Na^{22}$ activity

in the body and $Na^{22}$ concentration in serum. Others have reported half-times for urinary excretion two- to fourfold those for $Na^{22}$ concentration in serum.[2, 12] Such large differences were not observed in our studies.

The error in daily counts of radioactivity in the body can be estimated by an examination of the counts obtained during the period when the subject took only 1 mEq. of sodium per day (Fig. 8). The curve which represents $Na^{22}$ radioactivity in the body is relatively flat, and deviations of individual counts may be considered errors in counting. The maximum deviation from the assumed curve was less than 2.0 per cent.

The accuracy of calculated exchangeable sodium can be determined by referring also to the period of minimal sodium intake. After the initial drop in exchangeable sodium which followed the change from the normal to the 1 mEq. sodium diet, the exchangeable sodium should remain relatively constant. Therefore, variations from the average value (2,880 mEq.) for this period may be considered as errors in the calculated exchangeable sodium (Fig. 7). In no instance was the variation greater than 70 mEq. These variations represent a combination of inaccuracies in counts of radioactivity in the body and measurements of $Na^{22}$ activity in serum and sodium concentration in serum.

The limiting factor, which determines the length of the period in which the exchangeable sodium can be followed with the body radioactivity counter, is the absolute amount of $Na^{22}$ retained in the body. In the present study, measurements of $Na^{22}$ activity in the body and serum specific activity were being made after 38 days, when the amount of $Na^{22}$ which remained was only 3 $\mu$c. The effects of a specific dietary or drug regime, or the effects of a surgical procedure on exchangeable sodium in the body, therefore, can be followed continuously over prolonged periods of time with only an initial injection of a tracer dose of $Na^{22}$, and without the necessity for continuous urine collections.

The changes in exchangeable sodium with varying sodium intakes seen in normal subjects will be discussed elsewhere.[13] Similar studies are in progress on patients with cardiovascular and hepatic diseases.

## SUMMARY

A sensitive external gamma ray counter is described for clinical tracer studies of radioisotopes which do not concentrate specifically in any part of the human body. The detector is a 4 by 4 inch sodium iodide crystal, enclosed in a 5-inch-thick steel shield. The background counting rate, gamma ray counting efficiency, and photopeak efficiency of the body radioactivity counter are discussed, and values are given for the 1.28 mev. gamma rays of $Na^{22}$.

In a long-term $Na^{22}$ study of a normal subject, the body radioactivity counter measurements proved to be more accurate in following $Na^{22}$ retention in the body than the standard method of measurement of $Na^{22}$ excretion in urine, in that they accounted for both renal and extrarenal losses. The exchangeable sodium could be determined from the body radioactivity counter measurements and serum specific activities at any time, with better than 3 per cent accuracy. The exchangeable sodium was found to vary with sodium intake.

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# LABORATORY METHODS

## AN EXPERIMENTAL STUDY OF THE USE OF ROMANOWSKY STAINS FOR ESTIMATING THE HEMOGLOBIN CONTENT OF RED CELLS

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THE Romanowsky stains are probably the commonest group of stains used in the study of hematology, and it has long been the custom for hematologists to estimate the hemoglobin content of cells of the erythroid series by the amount of eosin taken up by them. Some have even claimed that eosin staining of any of the blood cells indicates that they may contain hemoglobin.[1] The aim of the present work has been to investigate these matters by microspectroscopy and to see how far the claims are justified. The following points have been investigated: (1) the staining properties of 2 different Romanowsky-type stains, (2) the amount of agitation during staining, (3) the staining time, (4) the position of the slide in the stain, (5) the packing of the cells on the slide, (6) the thickness and hemoglobin content of the cells, and (7) the validity of eosin staining as a guide to the hemoglobin content of blood cells.

### MATERIALS AND METHODS

Human blood smears were stained with either Wright's or McNeill's stain by the various techniques to be described. D.P.X.* was used as a mountant, since it is not fluorescent in the near ultraviolet.

The original absorption measurements were made on a microspectrograph with the use of a refracting microscope. The technique is described in full elsewhere.[2] Because of the inaccuracies inherent in any photographic method of measurement, and because of the chromatic aberration of refracting microscopes, a photometric system which incorporates a Burch[3] aspherized reflecting microscope was devised.[4]

Lillie and Roe[5] have carried out extensive studies of the Romanowsky stains and have shown that in such eosin azure stains both the eosin and the azure compounds have separately identifiable absorption bands in the green and red regions of the spectrum, respectively. Staining merely adds the absorption of eosin (525 $\mu$) to that of the Soret[6] band of hemoglobin (415 $\mu$) (Fig. 1). Immersion in methyl alcohol (the fixative and stain solvent) is known to have no effect on the Soret absorption of hemoglobin.[2, 4] The light absorption of a specimen is given by Beer's and Lambert's laws: $I = I_0 10^{-kcd}$ or in a more useful form: $kcd = \frac{\log I_0}{\log I}$, where k = a constant for the wavelength and compound under observation.

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$c$ = concentration, $d$ = thickness, $I$ = the light intensity transmitted, and $I_0$ = the light intensity incident on the specimen. In words, the concentration times the thickness of the specimen, i.e., the absolute amount of absorbing material, is proportional to the logarithm of the absorption of light at an absorbing wave length. Hence the ratios of the logarithms of the absorptions at their absorption maxima will give the ratios of the amounts of eosin and hemoglobin present in that part of the cell under examination in the microspectrophotometer.

## RESULTS

The earlier experiments were carried out with the photographic technique in which a refracting microscope is used, but it soon became obvious that the errors in the photographic technique were as great as the variation in the ratio $\frac{\log \text{absorption } 525\ \mu}{\log \text{absorption } 415\ \mu}$ between cells on the same slide. It was also discovered that this ratio depended very markedly on the wave length at which the microscope was focused. Thus for a given cell, with the microscope focused in green light, the ratio was 0.6, but when the microscope was focused in violet light the same cell gave a ratio of 1.3. However, if the microscope is always focused with monochromatic light of the same wavelength, the results are repeatable, although an absolute measure of the true absorption is obtainable only at the wavelength at which the microscope is focused. Thus, even a good apochromatic objective will not allow absolute absorption measurements to be made at widely different wavelengths. A full discussion of the problems involved is given elsewhere.[7] The use of a Burch aspheric reflecting microscope enabled this problem to be overcome, and a photoelectric technique markedly increased the repeatability of the measurements. All subsequent experiments were carried out with this latter technique, and Fig. 1 is the sort of curve that it was possible to obtain from a small area of an individual cell.

*A Comparison of Two Different Stains.*—In this group of experiments human blood smears were stained in an identical manner, with either McNeill's or Wright's stain. A measured quantity of stain was run on to the slide, and after 1 minute an equal quantity of distilled water was thoroughly mixed with the stain. After a further 5 minutes, the stain was tipped off, and the slide was rinsed 3 times in distilled water before being allowed to dry. The absorption of randomly selected cells on each slide was measured at 525 $\mu$ and 415 $\mu$, and the ratios were calculated. This ratio for McNeill's stain worked out at $1.58 \pm 0.08$. Similar measurements on slides stained with Wright's stain gave $0.90 \pm 0.11$. Clearly, the type of stain is of major importance in governing the amount of eosin taken up by red cells.

*The Effect of Agitation.*—In an attempt to improve the evenness of staining and the uniformity of uptake of eosin by red cells, blood films were stained for various periods of time, and very thorough agitation of the stain over the surface of the slide was maintained during the staining process. Cells on these slides were then measured as described and compared with cells from slides stained for identical periods of time, but with a more conventional degree of agitation. Two things were observed. The expected reduction in the standard

deviation of the ratio from one cell to another was obtained, and the uptake of stain was slower. Thus for 5 minutes with McNeill's stain the ratio was 1.28 ± 0.02, as compared with 1.58 ± 0.08. Similar results were obtained with other periods of time of staining.

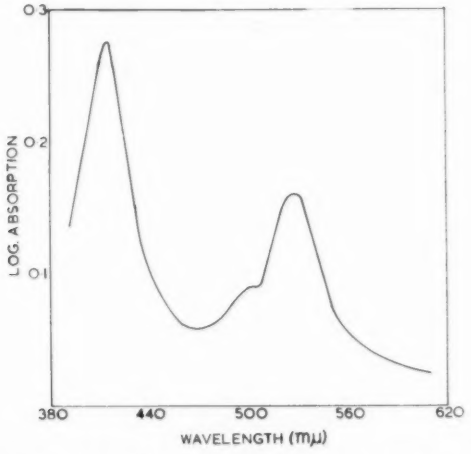


Fig. 1.—Absorption spectrum of a small area of an individual erythrocyte stained with Wright's stain which shows the eosin and Soret maxima.

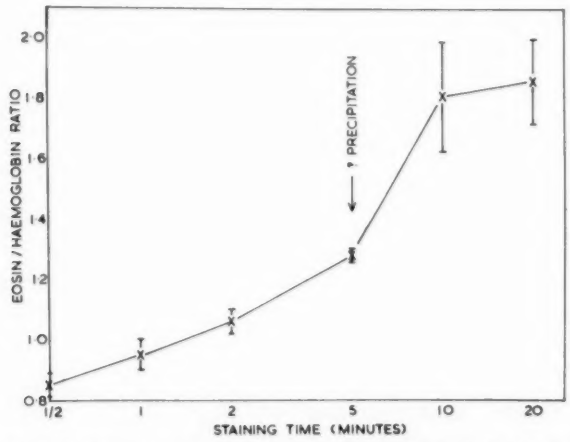


Fig. 2.—The effect of staining time on the eosin to hemoglobin ratio of stained human erythrocytes (McNeill's stain).

*Staining Times and the Uptake of Eosin.*—The length of staining, obviously an important factor in the determination of the uptake of eosin, was investigated. Slides stained conventionally, and those stained with vigorous agitation, were measured, and it was found that for the first 5 minutes the uptake of eosin was almost exactly logarithmic. The departure from this is less than the standard deviation of the individual results. Fig. 2 shows the results graphically. After 5 minutes, the scatter increased enormously, even in well-agitated slides. This was probably caused by precipitation of the stain.

*The Position of the Slide in the Stain.*—The marked effect of agitation on staining raised 2 possibilities. Either the agitation prevented the settlement of the precipitate on the slide, or the cells were maintained in contact with fresh stain. Slides were, therefore, mounted vertically and stained in a deep bath of Wright's stain. Table I shows that the uptake of stain is considerably more rapid and reaches a plateau at a fairly constant level between 2 and 5 minutes, after which no further stain is taken up for at least 20 minutes. This suggests that the increased but irregular staining, that occurs after 10 minutes with McNeill's stain, when the slides are mounted horizontally, is stain precipitation rather than true staining. It is difficult to understand why the depth of staining should be greater when the slides are mounted vertically, but the method is, in any case, of little practical value, as nuclear staining is not satisfactory with this technique.

TABLE I. THE UPTAKE OF EOSIN BY RED CELLS STAINED WITH WRIGHT'S STAIN SHOWN CONVENTIONALLY, AND IN A DEEP BATH WITH THE SLIDE IN A VERTICAL POSITION

| TIME (MINUTES) | HORIZONTAL STAINING (EOSIN:HB.) | VERTICAL STAINING (EOSIN:HB.) |
|---|---|---|
| ½ | 0.57 ± 0.04 | 0.91 ± 0.04 |
| 1 | 0.71 ± 0.03 | 1.47 ± 0.04 |
| 2 | 0.76 ± 0.05 | 1.82 ± 0.17 |
| 5 | 0.90 ± 0.11 | 2.27 ± 0.32 |
| 10 | 1.21 ± 0.10 | 2.00 ± 0.05 |
| 20 | 1.26 ± 0.08 | 2.00 ± 0.09 |

*The Packing of the Cells.*—Smears made deliberately uneven so as to produce crowded and sparse areas were examined to see if there was any relationship between the packing of the cells and the uptake of stain. No such relationship could be found. The variation in staining was much more dependent on the thoroughness of mixing and the degree of agitation.

*The Effect of Cell Thickness and Hemoglobin Concentration.*—The log absorption is proportional both to the concentration of the absorbing compound and to its thickness. As we did not measure the thickness of the smeared cells, these 2 factors could not be separated. The combined variation over the cells studied was large, but the ratio $\frac{\text{log absorption } 525\ \mu}{\text{log absorption } 415\ \mu}$ was quite independent of this factor. This means that the redness of a cell is related to the amount of hemoglobin looked through, and that it is impossible to differentiate between a thin cell with a high concentration and a thick cell with a low hemoglobin concentration.

## CONCLUSIONS

Our results show that eosin staining is a surprisingly reliable guide to the hemoglobin content of a red cell. McNeill's stain was found to be more reliable than Wright's, and, with the same stock solution of this stain and a carefully standardized staining procedure, a repeatability of better than 5 per cent was obtained. The following points are important in obtaining consistent results:

1. The duration of staining should be accurately controlled and should not exceed 5 minutes.

2. Mixing of the stain with distilled water should be thorough.

3. The degree of agitation during staining should be standardized and should be considerable.

We would like to thank Professor J. M. Yoffey for his encouragement and for the generous facilities he has provided.

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ERRATUM

In the article by Ludwig Kornel entitled "An Improved Rapid Method for Free and Conjugated 17-Hydroxycorticosteroids in Plasma," vol. 54, p. 666, October, 1959, the second line from the top should read, "values for the free 17-OHCS were found between 7.5 and 21.0 $\mu$g per 100 ml."

# AN ENZYMATIC SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF PHENYLALANINE IN BLOOD

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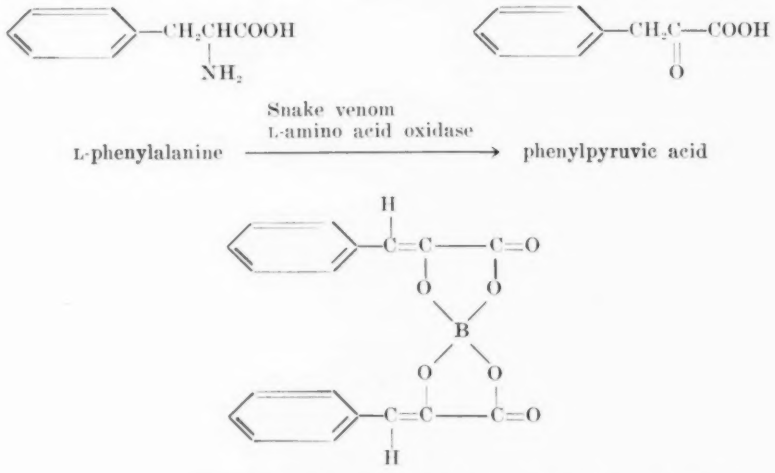
BETHESDA, MD.

THERE is a need for a simple rapid method to measure phenylalanine in serum or plasma, not only as an aid in the diagnosis of phenylketonuria, but also in the evaluation of the effectiveness of a diet low in phenylalanine in the treatment of patients with this condition. The method most commonly used to measure phenylalanine in plasma is that of Udenfriend and Cooper,[1] in which plasma is deproteinized and treated with *Streptococcus faecalis* to decarboxylate L-phenylalanine to phenylethylamine. The latter is then determined colorimetrically after reaction with methyl orange. Although this method has been used successfully by many workers,[2-6] it requires relatively large amounts of plasma and takes several hours to perform each set of analyses. Furthermore, extremely careful technique is necessary to avoid an appreciable and variable blank from methyl orange.

Another method for the determination of serum phenylalanine is that of Berry,[7] by paper chromatography. This method, although simple, has limitations in quantitative analysis inherent in any method which depends upon estimation of the intensity of colored spots on paper chromatograms.

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Received for publication June 22, 1959.

A simple rapid method is described which permits the analysis of plasma L-phenylalanine in 0.1 ml. of serum without deproteinization. The method utilizes L-amino acid oxidase from snake venom to oxidize L-phenylalanine to phenylpyruvic acid. In the presence of arsenate and borate ions the resulting α-keto acid is rapidly converted to an enol-borate complex which has a high absorption in ultraviolet light. Catalase is added to protect the α-keto acid from peroxide formed in the oxidative deamination by L-amino acid oxidase.

Enol-borate complexes have been employed by Knox and Pitt[8] to determine p-hydroxyphenylpyruvic acid oxidase activity in mammalian liver preparations. More recently these complexes have been used to measure the activity of several enzymes which either form or break down the aromatic α-keto acids related to phenylalanine, tyrosine, tryptophan, and histidine.[9]

## METHOD

*Reagents.—*

1. Phosphate buffer: 0.2M sodium phosphate buffer, pH 6.5.

2. Arsenate-phosphate buffer: 2.0M sodium arsenate was dissolved in the 0.2M phosphate buffer, and the final pH was readjusted to 6.5 with dilute HCl.

3. Borate-arsenate reagent: 1.0M borate, dissolved in 2.0M arsenate, pH 6.5, (61.8 Gm. boric acid + 624 Gm. sodium arsenate [$Na_2HAsO_4 \cdot 7H_2O$]) was adjusted to pH 6.5 with HCl and made up to 1 L.

4. Snake venom L-amino acid oxidase (the venom of *Crotalus adamanteus**): a suspension of dried venom in water was made which contained 10 mg. per milliliter. This was centrifuged, and the clear supernatant solution was removed and used. The enzyme solution is kept at 0 to 5° C. and remains active with little loss of activity for several days. The dry venom is stored in the refrigerator and maintains high enzyme activity for several months.

The activity of the L-amino acid oxidase preparation can be assayed by using 0.1 ml. of the standard phenylalanine solution in place of serum in the directions given below.

5. Catalase: crystalline beef liver catalase,† diluted 1:5 with 0.2M phosphate buffer, pH 6.5. The enzyme solution is kept at 0 to 5° C. and can be used for several weeks.

6. Standard phenylalanine solution: L-phenylalanine, 1 μM per milliliter (0.1 ml. contains 16.5 μg).

7. Blood serum: Blood is drawn and allowed to clot. (Some samples of heparinized plasma develop turbidity during analysis which makes them unsuitable for this determination.)

*Procedure.—*

Three 1.2 ml. quartz Beckman cuvettes with a 1 cm. light path are used for the determinations. The additions to each of the cuvettes are as follows:

| | $C_1$ | $E_1$ | $E_2$ |
|---|---|---|---|
| $PO_4$ buffer, 0.2M, pH 6.5‡: | 0.39 | 0.39 | 0.39 |
| Arsenate-phosphate, pH 6.5: | 0.5 | — | — |
| 1M borate in 2M arsenate: | — | 0.5 | 0.5 |
| Catalase, 1:5 dilution: | 0.01 | 0.01 | 0.01 |
| Venom, 10 mg. per milliliter: (Serum added later.) | 0.10 | 0.10 | 0.10 |

The contents are mixed and the duplicate experimental cuvettes, $E_1$ and $E_2$, are read against the control cuvette, $C_1$, at 308 mμ, in the Beckman spectrophotometer.§ If the addi-

*From the Ross Allen Reptile Institute, Silver Springs, Fla.

†From the Worthington Biochemical Corporation, Freehold, N. J.

‡If more than 0.1 ml. of serum is to be added, the volume of phosphate buffer must be reduced proportionately.

§Model DU with ultraviolet attachment.

tions have been made correctly, this reading should be approximately zero. Then 0.1 ml. of serum is added to cuvettes $C_1$, $E_1$, and $E_2$, mixed, and readings are taken at 1 or 2 minute intervals for 10 minutes at 308 mμ, to be certain that the enzyme activity is adequate, and that the reaction is essentially complete, as indicated by finding no further change in the optical density reading. Final readings are made at 308, 330, and 350 mμ at this time.

*Calculations.*—

Readings are taken at 330 and 350 mμ, as well as at 308 mμ, to correct for the absorption of the keto acids derived from tyrosine and tryptophan, since the latter 2 amino acids are also oxidized by the venom L-amino acid oxidase. The correction applied is based upon the relative absorption of the enol-borate complexes of these keto acids at 308, 330, and 350 mμ:

| | RELATIVE OPTICAL DENSITY READINGS AT DIFFERENT WAVELENGTHS (mμ) | | |
|---|---|---|---|
| KETO ACIDS OF | 308 | 330 | 350 |
| X = tyrosine | X | 0.60X | 0.06X |
| Y = phenylalanine | Y | 0.10Y | 0 |
| Z = tryptophan | 0.85Z | 1.41Z | Z |

Let the reading at 308 mμ = A; 330 mμ = B; 350 mμ = C

Then: $A = X + Y + 0.85Z$

$B = 0.6X + 0.1Y + 1.41Z$

$C = 0.06X + Z$

Solving these equations gives:

$$X = 2.38B - 0.238A - 3.16C \quad (1)$$

$$Y = 1.23A - 2.26B + 2.15C \quad (2)$$

$$Z = 1.19C - 0.14B + 0.014A = C - 0.06X \quad (3)$$

The absorption at 308 mμ caused by phenylpyruvic acid is given by solving for Y in equation (2):

$\frac{Y}{0.031} \times 10 =$ μg phenylalanine per milliliter of serum (if 0.1 ml. of serum were analyzed). One microgram phenylalanine under these conditions of 1.1 ml., etc., at 308 mμ reads 0.031 O.D. units.

By the same means, solving for X in equation (1) and dividing: $\frac{X}{0.045} \times 10 =$ μg tyrosine per milliliter of plasma (1 μg tyrosine under these conditions reads 0.045 at 308 mμ); and, similarly, using equation (3): $\frac{Z}{0.033} \times 10 =$ μg tryptophan per milliliter of serum (1 μg of tryptophan reads 0.033 at 350 mμ).

## RESULTS AND DISCUSSION

*Specificity.*—The L-amino acid oxidase of snake venom catalyzes the oxidation of a number of amino acids. However, only 4 of these are present in appreciable quantities in serum which yields α-keto acids with highly absorbing enol-borate complexes; these are phenylalanine, tyrosine, tryptophan, and histidine. Fortunately, histidine is not oxidized under the experimental conditions described above, and the relative contribution of each of the other 3 amino acids can easily be determined from the absorption of the combined complexes at 3 different wavelengths. Therefore, this method permits the simultaneous determination of phenylalanine, tyrosine, and tryptophan in serum. If the value of phenylalanine alone is desired, the correction for absorption caused by the keto acids of tyrosine and tryptophan would appear to be a disadvantage of the method. This is partly compensated for by the commercial availability of the

enzyme, its stability, and its high activity. Furthermore, the ease with which both phenylalanine and tyrosine can be measured is of special interest in some studies on phenylketonuria; for example, in the detection of the heterozygous carrier of this trait by the ratio between the levels of phenylalanine and tyrosine in the blood after the administration of phenylalanine as a tolerance test.[9]

The wide range of substrates which can be used with this enzyme has made it possible to extend this method to measure a number of other aromatic amino acid analogues and antimetabolites, such as p-fluorophenylalanine, m-tyrosine, monoiodotyrosine, mononitrotyrosine, and β-2-thienylalanine. These compounds are also rapidly oxidized by the L-amino acid oxidase of snake venom, and the resulting keto acid products can be measured as enol-borate complexes.[10]

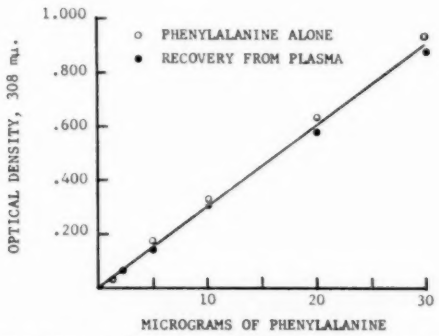


Fig. 1.—Graph of the optical density at 308 mμ versus concentration of phenylalanine when determined in standard solutions and when added to normal plasma. The linearity between optical density and phenylalanine concentration is demonstrated.

*Reproducibility and Accuracy.*—The method described is well suited to measuring the elevated blood level of phenylalanine found in untreated phenylketonuric individuals and to following the effectiveness of a diet low in phenylalanine in lowering the serum level in these individuals. The specificity of the absorption spectrum of the phenylpyruvic acid enol-borate complex and the ease and rapidity of the analysis permit a large number of determinations to be carried out within a short period of time. Fig. 1 demonstrates the linear relationship between phenylalanine concentration and optical density change between 2 and 30 μg analyzed alone and the recovery of phenylalanine added to normal plasma in this range. These results are in agreement with the theoretic values expected. However, the precision of the analytic method is lower in analyses of phenylalanine in normal serum than when the values are elevated as in serum of an individual with phenylketonuria. In normal serum the contributions of tyrosine and tryptophan to the reading at 308 mμ are appreciable. Even though duplicate pairs of analyses in a series of normal serum samples analyzed on successive days rarely disagreed by more than 7 per cent, larger aliquots of serum would be desirable if the method were to be used to measure, with high accuracy, the endogenous phenylalanine, tyrosine, and tryptophan levels. Attempts to carry out the analyses directly on 0.2 or 0.3 ml. of serum have been only partly successful, since a turbidity often occurs. For this reason,

several methods were tested to deproteinize the sample before analysis. Preliminary experiments have been made using perchloric acid precipitation followed by neutralization and the removal of $KClO_4$, as described by Segal, Blair, and Wyngaarden,[11] before enzymatic assay of blood pyruvate. This procedure for deproteinization appears to be satisfactory in eliminating the turbidity problem, and the solution which results can be used directly in the enzymatic assay.

*Phenylalanine Levels in Normal and Phenylketonuric Individuals.*—The serum phenylalanine levels of 30 adult blood bank donors in a nonfasting condition were found to have an average value of 1.55 mg. per cent (range, 0.84 to 2.64 mg. per cent). It is probable that the level in normal subjects in a fasting condition is somewhat lower than these values. In a smaller number of analyses in laboratory workers in a fasting condition, the group average is slightly lower than the above value.

The level of phenylalanine in a group of 10 phenylketonuric individuals on a regular diet was found to have an average value of 36.8 mg. per cent (range, 26.2 to 59.1 mg. per cent). These values in normal and in phenylketonuric people are in good agreement with those in the literature[7, 12-14] obtained by other methods. The wide difference in the levels of normal and phenylketonuric individuals makes the analysis of serum phenylalanine a valuable diagnostic procedure.

## SUMMARY

A simple enzymatic spectrophotometric method has been described for the quantitative determination of phenylalanine in serum. The method is based upon the measurement of the absorption of the enol-borate complex of phenylpyruvic acid generated enzymatically from phenylalanine by L-amino acid oxidase of snake venom. For elevated serum levels of phenylalanine the method is rapid, specific, and precise. It should be useful as a confirmatory test in the diagnosis of suspected phenylketonuria and in the evaluation of the effectiveness of a diet low in phenylalanine. The values obtained by this method agree well with those in the literature obtained by other techniques. Tyrosine and tryptophan are also determined by this method, and a suitable modification of the method is described which should allow an accurate estimation of these amino acids and phenylalanine in normal serum.

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**ERRATUM**

In the article by Mary S. McCall and Sue Keller, "An Abbreviated Method for Blood Volume Measurement Employing Radiochromium," vol. 55, p. 150, January, 1960, in Table I, the column under "Standard Method, Equilibration Time" should read:

30 minutes
1 hour
2 hours
2 hours
1 hour
15 minutes
15 minutes
15 minutes
15 minutes
15 minutes.